TEXAS REPORTS on BIOLOGY and MEDICINE

VOLUME 11, NUMBER 2 SUMMER, 1953

Copyright 1953 by the Board of Regents of The University of Texas

Contents

Page

Amino Acid Metabolism in Vitamin A Deficiency: I. Free Amino Acids in Tissues of Normal and Deficient Rats, *Mohammed Abdulnabi and J. G. Bieri* 181

Amino Acid Metabolism in Vitamin Deficiency: II. The Glutamic-Aspartic Transaminase Activity in Tissues from Normal and Deficient Rats, *Mohammed Abdulnabi and J. G. Bieri* 196

Appartus for Stabilizing the Chemical and Physical Environment of Certain Free-Living Cells, *Iben Browning and Lloyd S. Lockingen* 200

The Alteration of Plasma and Aortal Lipids by Adenosine-5-Monophosphate, *William W. Calhoun, Lawrence J. Milch, Robert F. Redmond, and the Cardiovascular Research Group* 207

The Ocular Changes in Diabetes and Their Effect on the Patient's Vision, *Frederick C. Cordes* 213

Free Amino Acids in Serious Fluids, *J. P. Ellis, W. W. Nowinski, and J. G. Bieri* 231

The Evolution of Esophagoplasty, *Wesley Fry* 236

Observations on the Behavior of Exfoliated Cells from Breasts of Dogs in Tissue Culture, *W. C. McCormick, Dudley Jackson, and C. M. Pomerat* 257

Effects of Increasing Doses of Morphine, Dromoran and Sodium Amytal on Rat Tissues *in vivo* and *in vitro*, *W. C. McCormick and W. T. Kniker* 274

The Use of Yolk Sac Cultivated Bacteria in Bacterial Studies, *George F. McKenna and Alfred Taylor* 283

Isoniazid in the Treatment of Tuberculous Meningitis, *John W. Middleton and A. N. Longfield* 287

The Sensory Influences of the Skin, *M. F. Ashley Montagu* 291

[Contents Continued on Next Page]

Page

Clinical Management of the Irritable Colon and Diverticulosis, *James W. Morgan* 302

Thermal Stress and Tissue Culture: I. The Effect of Rapid Freezing and Thawing on Chick Embryonic Epidermis, Skeletal Muscle and Spindle Cells, *C. M. Pomerat and Robert B. Lewis* 307

Thermal Stress and Tissue Culture. II. The Effect of Rapid Freezing and Thawing on Human Newborn and Adult Skin, *C. M. Pomerat and Robert B. Lewis* 333

Comparison by Electron Microscopy of the Ntaya and Zika Viruses, *Reginald L. Reagan and A. L. Brueckner* 347

Paper Electrophoresis of Hemoglobin as a Practical Method of Differentiating Various Types of Sickle Cell Disease and of Hemoglobin "C" Trait, *Rose G. Schneider* 352

The Etiology of Nutritional Muscular Dystrophy, *Ira R. Telford* 366

Calling Attention To 372

Edited at The University of Texas Medical Branch, Galveston

AMINO ACID METABOLISM IN VITAMIN DEFICIENCY:

I. Free Amino Acids in Tissues of Normal and Deficient Rats

Mohammed Abdulnabi and J. G. Bieri*

Outside of its role in the mechanism of vision, the biochemical function of vitamin A is still unknown. However, it is known to be concerned with various broad physiological activities, the most outstanding of which is the maintenance of the integrity of the epithelial tissues (Wolbach and Howe, 1925). Just how vitamin A maintains the integrity of the epithelial tissues has not been elucidated. Mason and Ellison (1935) stated that the inability of vitamin A deficient rats to produce normal, mucified epithelial cells indicated a definite alteration in the physiological state of the cell. These workers suggested that vitamin A may play a role in protein metabolism or synthesis within the epithelial cells. Since little is known of possible changes in protein metabolism as a result of vitamin A deficiency, a series of studies into this problem has been carried out. In the present paper, the free amino acid patterns in testes and submaxillary glands, organs known to show distinct pathological changes in vitamin A deficiency, have been determined. Blood serum also has been analyzed since no detailed accounts of the free amino acids in rat blood, as determined by paper chromatography, have appeared. In addition, a quantitative investigation by microbiological assay methods of the free amino acids in several rat tissues is reported.

I. Paper Chromatographic Analysis

Experimental. Weanling, male Holtzman rats were divided into two groups and pair-fed either the U.S.P. vitamin A assay ration or a purified, vitamin A-free diet (Bieri and Sandman, 1951). Control animals were fed one drop of cod-liver oil daily. When the first stage of vitamin A de-

* From the Department of Biochemistry and Nutrition, The University of Texas Medical Branch, Galveston. The material in this paper is from a thesis submitted to the Graduate School by Mohammed Abdulnabi in partial fulfillment of the requirements of the Doctor of Philosophy degree. This work was supported, in part, by the Williams-Waterman Fund of the Research Corporation. Received for publication January 30, 1953.

ficiency appeared in an experimental animal, both the depleted rat and its control were fasted twelve hours. Under ether anesthesia, they were bled to death from the carotid artery. The testes and submaxillary glands were quickly homogenized with 2–3 ml. of water. The homogenates, and serum, were immediately deproteinized with ethanol and the amino acids extracted by the procedure of Awapara (1948). The protein-free extracts were adjusted to pH 7–8 with dilute ammonia and desalted by the electrodialysis method of Consden, Gordon, and Martin (1947). With our apparatus, the initial potential of 25–35 volts rose to a maximum of 95 volts when desalting was completed, usually in 6–10 minutes. Under these conditions no conversion of arginine to ornithine occurred, as found by Stein and Moore (1951) with longer desalting periods. The desalted samples were stored at 5° C. until analyzed. Prior to analysis, the extracts were concentrated to 0.2 ml. in a 50° C. water bath. They were then analyzed by the two dimensional ascending technique of Williams and Kirby (1948). The samples were applied to Whatman No. 1 filter paper in portions of 10 microliters. Each sample represented 0.4–0.6 ml. of serum, 0.3–0.5 gm. of testes, or 0.05–0.10 gm. of submaxillary glands from individual animals. The chromatograms were run at 26–28° C. using phenol-water (75:25 by volume) as the first solvent, and 2,4–lutidine–water (65:35 by volume) as the second solvent. Solvents were removed by drying for one hour in a forced air oven at 85° C. When compared with chromatograms dried at room temperature, no significant destruction of amino acids by the heat-drying was apparent (Brush, Boutwell, Barton, and Heidelberger, 1951). The amino acids were located by spraying the sheets with 0.2 per cent ninhydrin in water-saturated butanol, followed by heating for 5–7 minutes at 85° C. The spots were located and marked with the aid of a fluorescent X-ray viewer.

The identification of the spots was made from a map containing 28 compounds, similar to that of Dent (1948). The map used in this investigation is represented in figure I. It was constructed by chromatographing standard solutions of amino acids by the two dimensional ascending technique described above. Verfication of spots in cases of questionable identification was made by applying to the paper a small amount of the amino acid involved in addition to a portion of the sample in question before chromatographing. If the added amino acid was superimposed on the unidentified spot, the identity was then assumed to be established. It is recognized, however, that this procedure does not constitute positive identification.

Although the majority of the chromatograms from desalted samples were clear and showed distinct spots, a few chromatograms occasionally appeared irregular and revealed overlapping spots. Thus, methionine and valine, spots 9 and 10 on the map, frequently were not separately distinguishable. Similarly, spot 11 represents both leucine and isoleucine. Spots 4 and 5 both correspond to glutamine. Early in this study, β-alanine was thought to be present in some tissues, but the spot in question was found to be part of the larger glutamine spot.

Results. A list of the compounds, and the number of animals in which each amino acid occurred, is given in Table 1. A total of 25 different spots was detected in the three tissues analyzed. The gross examination of Table 1 reveals that the compounds may be grouped as follows: 1. Amino acids, or their derivatives, which were found in all samples studied. These include aspartic acid, glutamic acid, α-alanine, glycine, serine, methionine (or valine), leucine, phenylalanine, and taurine. 2. Compounds that were found in all tissues but appeared less frequently in one or the other tissue. These include glutamine, threonine, proline, arginine, lysine, glutathione, ethanolamine phosphoric acid, and γ-aminobutyric acid. 3. Compounds that were not present in all tissues. These include asparagine, fast peptide, "under-taurine," "under-tyrosine," and hydroxyproline. 4. A single amino acid, α-aminobutyric acid, can be considered differently from other compounds because not only was it infrequent in its appearance, but it was absent from all control samples of testes and submaxillary glands.

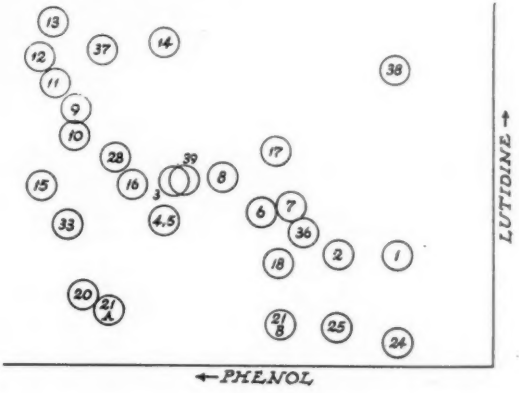


FIG. I. Map used for identifying amino acids.

1. Aspartic acid
2. Glutamic acid
3. Alanine
4, 5. Glutamine
6. Glycine
7. Serine
8. Threonine
9. Methionine
10. Valine
11. Leucines
12. Phenylalanine
13. Tryptophan
14. Tyrosine
15. Proline
16. Hydroxyproline
17. Taurine
18. Asparagine
20. Arginine
21. Lysine
24. Gluthathione
25. Ethanolamine Phosphate
28. α-aminobutyric acid
33. γ-aminobutyric acid
36. Isoserine
37. 3, 5-diiodotyrosine
38. Dihydroxyphenylalanine
39. Homoserine

Although the general pattern of ninhydrin-reacting substances was quite similar for the different tissues investigated, a large number of the compounds varied considerably in their frequency of appearance in one tissue to another. Such compounds are represented by those classified under (2). Furthermore, several compounds appeared infrequently in the same tissues from different rats. A few amino acids, for example, α-aminobutyric acid, γ-aminobutyric acid, hydroxyproline, and asparagine were found infrequently and might not have been detected if a large number of animals had not been used.

TABLE 1

Occurrence of Free Amino Acids in Serum, Submaxillary Glands and Testes of Normal and Vitamin A Deficient Rats

Amino Acid*	Serum		Submaxillary Gland		Testes	
	Deficient (15)†	Normal (17)	Deficient (17)	Normal (15)	Deficient (21)	Normal (19)
1. Aspartic acid ...	15	17	14	9	19	17
2. Glutamic acid ..	15	16	15	13	20	18
3. Alanine	12	15	14	12	18	16
4, 5. Glutamine	10	16	6	7	11	16
6. Glycine	15	17	15	12	21	19
7. Serine	12	14	12	7	13	9
8. Threonine	5	8	6	4	3	3
9. Methionine	14	17	12	10	15	15
10. Valine	10	10	6	7	13	13
11. Leucines	13	16	10	10	13	15
12. Phenylalanine ..	8	8	8	7	13	11
14. Tyrosine	7	6	3	2	11	12
15. Proline	13	15	7	6	13	15
16. Hydroxyproline..	1	0	0	1	0	0
17. Taurine	9	10	12	9	19	18
18. Asparagine	0	0	1	2	0	0
20. Arginine	10	10	6	2	11	13
21. Lysine	7	5	4	4	9	12
24. Glutathione	2	2	2	3	13	8
25. Ethanolamine Phosphate	1	2	14	11	20	18
27. Peptide	0	0	11	9	21	18
28. α-aminobutyric acid	4	4	1	0	2	0
33. γ-aminobutyric acid	4	3	2	2	13	13
35. Under tyrosine..	4	2	0	0	0	0
B. Under taurine ..	0	0	0	0	4	4

* Numbers refer to identification of spots in Fig. 1.
† Numbers in parentheses indicate number of animals analyzed.

Serum: A typical chromatogram of serum from a normal rat is shown in figure II. Chromatograms from deficient animals were strikingly similar, so that no effect on the free amino acids as a result of vitamin A deficiency could be said to occur. About eighteen spots were generally found in the serum. Most of these spots corresponded to the usual natural amino acids. Spots corresponding to ethanolamine phosphoric acid, α-aminobutyric acid, γ-aminobutyric acid and hydroxyproline appeared in only a few samples.

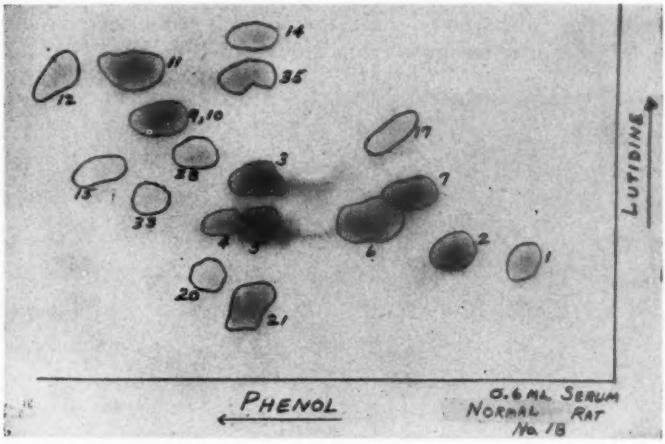


FIG. II. Chromatogram of free amino acids in normal rat serum. (See Fig. I for identification of spots. Spot 35 is unidentified.)

Of particular interest was the finding in six serums of an unidentified purple spot directly below tyrosine (Fig. II). In some cases, this spot was as intense in color as was the tyrosine spot. From the position of this spot, it appeared that it might have a structure closely related to tyrosine. Hence, two available compounds, 3,5-diiodotyrosine and dihydroxyphenylalanine (Dopa) were chromatographed in an attempt to identify the unknown spot. That this spot was neither of these two tyrosine derivatives was shown by the positions of these two compounds on the chromatograms (See map, Fig. 1).

Testes: A typical chromatogram from normal rat testes is shown in figure III. Chromatograms from normal and deficient

rat testes were also almost identical. About twenty spots were generally found in testes. As in the serum, the majority of these spots corresponded to the usual natural amino acids or their derivaties. The occurrence of amino acids in tests was, in general, more predominant and their spots more intense than those in the submaxillary glands or serums. Ethanolamine phosphoric acid was found in practically all samples, and γ-aminobutyric acid in most of them. The peptide (No. 27) described by Fuerst, Landua, and Awapara (1950) was almost always present. An unidentified, bright blue spot under taurine, and above serine, appeared when a chromatogram was erroneously heated at 120° C., after treatment with ninhydrin (Fig. III). This spot

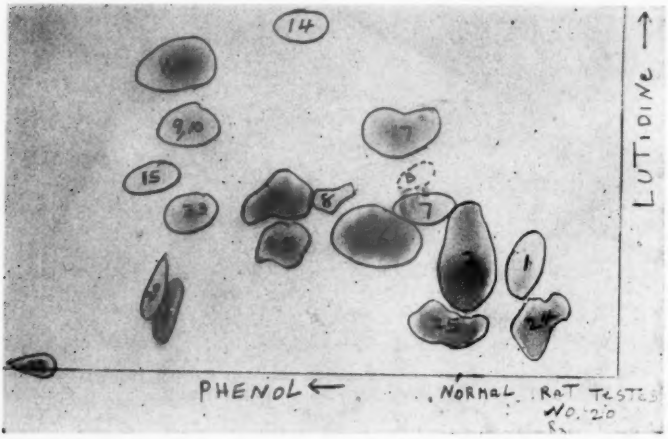


FIG. III. Chromatogram of free amino acids in normal rat testes. (See Fig. I for identification of spots. Spot "B" is unidentified.)

was later found in testes from four normal and four deficient rats. The color would develop only at this higher temperature. From the position of this spot, it appeared that it might be a short chain amino acid or a derivative related to serine, glycine, or taurine. Homoserine and isoserine were chromatographed to see if the unknown spot corresponded to either of these compounds. As is shown in the map, figure I, spots 36 and 39 correspond to isoserine and homoserine respectively and their positions are quite distinct from that of the unknown spot under

taurine. Furthermore, the unknown compound was a distinct blue while that of homoserine was purple and in the case of isoserine, yellow-brown.

Submaxillary glands: A chromatogram of this tissue from a normal rat is represented in figure IV. The chromatograms from this tissue were considerably more variable than from the serums or testes. Generally, only 8–10 spots appeared from any one sample. Since the glands from the animals were fairly uniform in weight, the observed variations in amino acids most probably represent individual variations among animals. In addition to the usual amino acids, ethanolamine phosphoric acid and peptide (No. 27) appeared in more than half of the samples. As with the other tissues studied, no effect of vitamin A deficiency on the ninhydrin-reacting substances in the submaxillary glands was apparent.

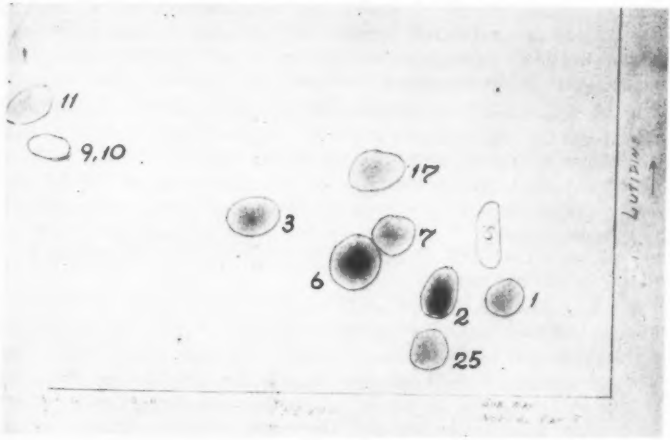


FIG. IV. Chromatogram of free amino acids in normal rat submaxillary glands. (See Fig. I for identification of spots. Spot "S" is salt.)

II. Microbiological Analysis

Experimental. The preparation of the normal and deficient animals was similar to that in the preceding section, except that the U.S.P. vitamin A assay ration was used exclusively. When the first stage of vitamin A deficiency appeared in an experimental animal, usually in 18–25 days (body weight 110–140 gm.), both the depleted rat and its control were fasted

24 hours. The animals were then killed by decapitation and the kidneys, testes and medial and right lobes of the liver excised and prepared by a modification of the method of Schurr, Thompson, Henderson, Williams, and Elvehjem (1950). Two or three grams of liver, and both kidneys, from each animal were treated separately, while the testes of two deficient or normal animals were combined. After removal from the animal, the tissues were immediately blotted with filter paper, weighed and dropped into homogenizing tubes containing 0.013 N. acetic acid in a boiling water bath. The time required after decapitation to remove and weigh the tissues and place them in the hot acetic acid was 3 to 4 minutes. Schurr, Thompson, Henderson, and Elvehjem (1950) showed that no apparent change in free amino acids in tissues of rats occurred within 5 minutes after death. The amounts of acetic acid used were 3 ml./gm. of liver or kidney and 1.5 ml./gm. of testes. After 4 minutes in the boiling water bath, the tissues were homogenized thoroughly while hot. The samples were then replaced in the boiling water bath for another 4 minutes with constant stirring. After centrifuging, the supernatants were deproteinized with tungstic acid. One-tenth volume of 0.6 N. sulfuric acid was added for the kidney extracts and 0.2 volumes for the liver and testes extracts. This was followed by 0.2 ml. of 10 per cent sodium tungstate for each gram of tissue. All samples were then stirred thoroughly and permitted to stand for 20 minutes. After centrifuging, the clear supernatants were frozen at —10° C.

Blood serums were deproteinized by the method of Hier and Bergeim (1945) and the filtrates frozen at —10° C. until analyzed. In order to have a sufficient amount of sample for the microbiological analysis, the blood filtrates of three animals were pooled and adjusted to pH 6.8–7.0 with 0.5 N. sodium hyroxide. The tissue filtrates were adjusted to the same pH in a similar manner. The final volumes of samples per gram of tissue (or per ml. serum) were 10 ml. for the kidneys, 5 ml. for the livers, and 3 ml. for the serums and testes.

The microbiological assay method employed here was that of Henderson and Snell (1948). The only changes in technique were those necessitated by the reduction in final volume in each assay tube from 2.0 to 0.5 ml. The composition of the basal medium was that proposed by Henderson and Snell (1948), but in the actual assay single strength medium was used rather than double strength. The amino acids not determined were made up in a dry solid mixture. The amino acids to be determined, namely, lysine, proline, tyrosine, valine, leucine, phenylalanine, arginine, threonine and methionine, were made up as nine solid mixtures. Each mixture contained eight amino acids exclusive of the one to be determined. Due to the large blank values encountered initially in the assays for methionine, this amino acid was not determined in the samples analyzed.

For these analyses a "composite standard," a dry mixture of amino acids, was employed to establish the standard curves. This standard mixture had the same composition as that proposed by Schurr, Thompson, Henderson, and Elvehjem (1950) with the exception of lysine which was made to

provide in the final dilution 20 mcg./ml. rather than 40. Standards, samples and water were dispensed with the Cannon automatic dispenser from an all-glass vessel. After dispensing the inoculated media, the assay tubes were incubated at 37° C. for 65–72 hours in a humidified incubator. The lactic acid produced was titrated electrometrically directly in the assay tubes with 0.04 N. sodium hydroxide. The Cannon automatic titrator was employed and the titration procedure and apparatus used were similar to those adapted by Henderson, Brickson, and Snell (1948).

To determine the reliability of the microbiological assay method employed, amino acid recoveries were performed on extracts from the serum and liver of a stock rat. Recoveries of added amino acids ranged from 99–111 per cent, with the exception of arginine. The recovery of arginine from liver filtrates was unsatisfactory due to the very low concentration of this amino acid in this tissue. With the amounts of filtrate used, the growth response of the microorganism to arginine was frequently too small to be reliable. A similar situation was found for this amino acid in testes. Consequently, only a few determinations of arginine in liver and testes are reported.

Since only small quantities of tissue extracts were obtained from individual rats, duplicate levels of 0.05, 0.10, and 0.15 ml. of each extract were assayed for the eight amino acids to be determined. Agreements among the three levels was generally ±11 per cent. Occasional individual values that were considerably out of line were discarded.

Results. A summary of the determinations of free amino acids in all tissues analyzed is presented in Table 2. It is apparent from this table that large variations are encountered in the values of each single amino acid in any given tissue whether from normal or deficient animals. Furthermore, the average amount of each amino acid tends, in general, to differ from one tissue to another in both normal and deficient animals. This variation was exemplified in practically all amino acids with the exception of lysine, which gave similar values for serum, liver, and kidney. Except for proline and arginine, the concentration of each amino acid in the kidneys was higher than in the other tisues investigated. Proline was present in testes in higher concentration than in kidney, liver, or serum. The average amount of arginine in serum was greater than in the other tissues studied.

As for the total amounts of all amino acids determined, the kidney had the greatest concentration, while liver had greater amounts than did serum or testes. The average values for normal serum, liver and kidney were greater than those for the

TABLE 2

Free Amino Acids in Tissues of Normal and Vitamin A Deficient Rats. Values Expressed as mcg./gm. Fresh Tissue, or mcg./ml. Serum. Figures in Parentheses Indicate Range of Values.

Amino Acid	Serum		Liver		Kidney		Testis	
	Deficient	Normal	Deficient	Normal	Deficient	Normal	Deficient	Normal
Arginine	27.1±10.2* (13.5—38.5)	38.0±10.8 (22.8—47.0)	4.5± 3.3 (2.0—10.8)	4.0± 1.6 (2.5— 6.2)	19.1± 8.7 (11.0—33.0)	19.8±14.5 (5.0—43.7)	3.8± 1.6 (2.0— 5.5)	2.0†
Threonine	36.4±19.7 (12.6—60.8)	60.6±21.0 (36.7—87.6)	42.0±11.2 (28.0—59.0)	78.6± 5.3 (73.0—86.3)	97.5±27.6 (54.0—130.0)	133.5±32.1 (90.5—182.0)	13.7± 2.5 (10.5—17.0)	10.3± 1.5 (9.0—12.0)
Valine	20.4± 3.7 (17.0—23.9)	21.6± 3.0 (18.6—26.6)	41.1±14.3 (21.7—59.5)	38.9±11.7 (25.0—56.0)	56.6±12.2 (43.0—81.0)	57.0±14.1 (43.0—86.5)	23.0± 3.6 (18.0—26.0)	16.1± 1.5 (14.0—17.3)
Leucine	28.6± 3.1 (25.0—32.1)	29.9± 2.2 (27.7—32.1)	58.5±14.3 (32.5—78.0)	53.0± 7.0 (41.0—64.7)	81.2±11.2 (67.5—102.7)	82.8±13.4 (60.0—99.0)	38.6± 3.0 (35.3—42.5)	27.0± 1.2 (25.3—28.0)
Phenylalanine	16.5± 1.7 (14.8—18.2)	21.4± 0.9 (19.6—23.1)	24.2± 3.9 (16.0—28.0)	25.7± 2.8 (21.3—29.0)	37.6± 7.1 (25.3—48.0)	40.9±13.8 (26.3—57.0)	19.8± 1.8 (16.7—21.3)	7.9± 0.9 (7.3— 8.5)
Tyrosine	17.7± 1.8 (15.8—19.2)	16.6± 1.7 (14.4—18.7)	35.0± 5.1 (26.0—41.7)	28.6± 5.7 (21.7—38.7)	52.8±12.7 (29.0—64.0)	49.4±12.8 (33.0—71.3)	23.7± 3.6 (20.3—29.0)	10.1± 1.1 (9.3—11.7)
Lysine	43.4± 0.2 (43.2—43.6)	44.5± 5.5 (39.7—52.2)	42.1±10.6 (26.5—56.0)	45.6± 9.6 (32.3—58.7)	44.0± 5.6 (34.5—50.3)	54.0± 9.6 (42.5—70.0)	37.8± 3.6 (34.0—42.0)	35.6± 2.0 (32.0—38.0)
Proline	8.8± 4.1 (4.7—12.9)	17.8± 1.3 (16.5—19.1)	19.8± 5.7 (13.0—30.0)	15.1± 4.0 (7.3—20.0)	34.9± 9.8 (20.0—47.5)	39.3±13.4 (22.5—61.0)	60.7± 6.6 (51.5—70.0)	67.3±12.6 (52.0—82.5)
Total	198.9	250.4	267.2	289.5	423.7	476.7	221.1	176.3

* Standard deviation.
† One determination only.

deficient tissues, while the opposite was true for testes. That each tissue had its characteristic quantitative pattern of amino acids is in confirmation of the qualitative studies by paper chromatographic analysis, i.e., each tissue was distinguished by a slightly different distribution of amino acids.

It has already been mentioned that there were no significant differences in the qualitative picture of amino acids from normal and deficient animals. The quantitative studies showed no difference between normal and deficient rats for most of the amino acids. However, statistical analysis revealed that the higher values of threonine in normal liver and of phenylalanine and tyrosine in deficient testes were highly significant. The P values were less than 0.001 for threonine and less than 0.01 for both tyrosine and phenylalanine. These are the only instances in which there were significant differences in amino acids between normal and deficient tissues.

Discussion. As was shown in the chromatograms, the general pattern of ninhydrin-reacting substances was quite similar for different tissues. Aspartic acid, glutamic acid, alanine, glycine, serine, methionine (or valine), leucine and taurine were found in practically all of the samples analyzed. The consistent occurrence of the first four of these amino acids is in agreement with the studies made by Awapara, Landua, and Fuerst (1950), who found aspartic and glutamic acids, alanine, and glycine in practically all of the eight rat organs they investigated. Tissues differed from each other, however, in the frequency of appearance of various other amino acids.. Such differences among tissues may be considered as reflections of the different metabolic patterns in any single tissue. The number and distribution of spots in serum were similar to those reported for human plasma (Agren and Nilsson, 1949; Crumpler, Dent, and Lindan, 1950) and for dog plasma (Flock, Mann, and Bollman, 1951). Of interest is the occurrence in six serum samples of the unidentified purple "under tyrosine" spot. No reports of a similar spot have appeared.

Besides the usual (i.e., frequently occurring) natural amino acids, ethanolamine phophoric acid and the "fast peptide" were found in both testes and submaxillary glands. A similar finding was reported by Awapara, Landua, and Fuerst (1950). The

blue spot "under taurine," found in testes, has not been reported previously.

The total average amount of free amino acids in normal rat serums, by microbiological assay, was higher than that reported for human serum (Hier and Bergeim, 1946; Harper, Hutchin, and Kimmel, 1952) and dog plasma (Hier and Bergeim, 1945; Hier and Bergeim, 1946). This total agreed closely, however, with that reported by Henderson, Schurr, and Elvehjem (1949), for the rat. The total amount of free amino acids in the latter case was 260.9 mcg./ml. for fasted rats, compared to 250.4 mcg./ml. for the animals analyzed in the present studies. The single average values of arginine, valine, and tyrosine were substantially the same. Threonine, leucine, and phenylalanine values were considerably higher (44–88 per cent) in the present investigation, while lysine and proline were lower (102–62 per cent) than those reported by the Wisconsin workers. The fluctuation of single amino acid values from one sample to another was noticeable in all the studies quoted above. Lysine, for example, had a two-fold variation in separate studies by the same group (Henderson, Schurr, and Elvehjem, 1949; Schurr, Thompson, Henderson, and Elvehjem, 1950).

The free amino acid concentrations in liver were, in general, lower than those reported by Schurr, Thompson, Henderson, Williams, and Elvehjem (1950), or by Solomon, Johnson, Sheffner, and Bergeim (1951). Similarly, the concentrations in kidney were lower than those found by the latter investigators. If age, strain, and weight of animals are factors which may contribute to the difference in amino acid values, as Schurr, Thompson, Henderson, Williams, and Elvehjem (1950) have stated, then the low values obtained in these studies may be explained on this basis. In both of the studies cited, the animals were considerably older and larger than those used in the present studies.

It appears from the results of our investigation that the qualitative picture of free amino acid in rats as obtained by the paper chromatographic technique is not altered by vitamin A deficiency. Although the results of the microbiological determinations supported those of the paper chromatographic analyses in most cases there were, however, a few exceptions which appear to be of interest. The increases in phenylalanine and tyrosine

concentrations in testes of deficient animals were significant. Of these two amino acids, phenylalanine is essential to the rat. The fact that tyrosine is formed in the body from phenylalanine may lead to the speculation that the increase in tyrosine concentration in deficient testes is a result of phenylalanine accumulation in this organ.

The possibility that vitamin A may be involved in the metabolism of the amino acids threonine, phenylalanine, and tyrosine is suggested by the above results. In a study such as this, however, care must be taken that any changes produced in the tissues are a specific result of insufficient vitamin A and are not due to a deficiency of several nutrients through general inanition. Mason (1933) elaborated on this point and compared the changes produced by vitamin A deficiency and by starvation. He further showed that in vitamin A deficiency lesions in the testes appear as soon as xerophthalmia is evident. In the present study, animals were sacrificed before advanced stages of deficiency set in and before the food intake had decreased sharply.

The considerable variation of many of the free amino acids in tissues as found in this study, and as found by other investigators (above), suggests that the concentrations of free amino acids in tissues are subject to considerable fluctuation and are probably affected by numerous metabolic factors.

Summary

Analysis by paper chromatography of the free amino acids in blood serum, testes and submaxillary glands of normal and vitamin A deficient rats did not reveal any differences due to a lack of the vitamin. Two unidentified spots were detected, one "under taurine" in testes and the other "under tyrosine" in serum. Considerable variation in the occurrence of many amino acids in the same tissue from similar animals was apparent.

The quantitative picture of most free amino acids is essentially not altered from normal by avitaminosis A, using the microbiological assay procedure. The amount of threonine was higher in normal livers than in deficient livers. An increased concentration of phenylalanine and of tyrosine in deficient testes was also found. In general, for any particular amino acid, a

comparison of its concentration in the same tissue from different animals revealed large variations.

Acknowledgments. The authors wish to acknowledge the helpful suggestions of Dr. L. M. Henderson concerning various techniques of the microbiological assay. They are also gratefully indebted to Dr. H. B. Lewis for a sample of isoserine, and to Dr. M. D. Armstrong for a sample of homoserine.

REFERENCES

Ägren, G., and T. Nilsson: Paper chromatographic analysis of amino acids and other ninhydrin-reacting substances in deproteinized human plasma. *Acta Chem. Scand.*, 3: 525–538, 1949.

Awapara, J.: Application of paper chromatography to the estimation of free amino acids in tissues. *Arch. Biochem.*, 19: 172–173, 1948.

Awapara, J., A. J. Landua, and R. Fuerst: Free aminoethyl phosphoric ester in rat organs and human tumors. *J. Biol. Chem.*, 183: 545–548, 1950.

————: Distribution of free amino acids and related substances in organs of the rat. *Biochim. et Biophys. Acta*, 5: 457–462, 1950.

Bieri, J. G., and R. P. Sandman: Comparative utilization of carotene administered orally and parenterally. *Proc. Soc. Exptl. Biol. Med.*, 77: 617–619, 1951.

Brush, M. K., R. K. Boutwell, A. D. Barton, and C. Heidelberger: Destruction of amino acids during filter paper chromatography. *Science*, 113: 4–6, 1951.

Consden, R., A. H. Gordon, and A. J. P. Martin: The identification of lower peptides in complex mixtures. *Biochem. J.*, 41: 590–596, 1947.

Crumpler, H. R., C. E. Dent, and O. Lindan: The amino acid pattern in human fetal and maternal plasma at delivery. *Biochem. J.*, 47: 223–227, 1950.

Dent, C. E.: A study of the behavior of some sixty amino acids and other ninhydrin-reacting substances on phenol-collidine filter paper chromatograms, with notes as to the occurrence of some of them in biological fluids. *Biochem. J.*, 43: 169–180, 1948.

Flock, E. V., F. C. Mann, and J. L. Bollman: Free amino acids in plasma and muscle following total removal of the liver. *J. Biol. Chem.*, 192: 293–300, 1951.

Fuerst, R., A. J. Landua, and J. Awapara: The presence in animal organs and human blood of a peptide detected by paper chromatography. *Science*, 111: 635, 1950.

Harper, H. A., M. E. Hutchin, and J. T. Kimmel: Concentration of nineteen amino acids in plasma and urine of fasting normal males. *Proc. Soc. Exptl. Biol. Med.*, 80: 768–771, 1952.

Henderson, L. M., W. L. Brickson, and E. E. Snell: A micromethod for the microbiological determination of amino acids. *J. Biol. Chem.*, 172: 31–38, 1948.

Henderson, L. M., P. E. Schurr, and C. A. Elvehjem: The influence of fasting and nitrogen deprivation on the concentration of free amino acids in rat plasma. *J. Biol. Chem.*, 177: 815–823, 1949.

Henderson, L. M., and E. E. Snell: A uniform medium for determination of amino acids with various microorganisms. *J. Biol. Chem.*, 172: 15–29, 1948.

Hier, S. W., and O. Bergeim: Microbiological determination of free leucine, isoleucine, valine, and threonine in dog plasma. *J. Biol. Chem.*, 161: 717–722, 1945.

————: The microbiological determination of certain free amino acids in human and dog plasma. *J. Biol. Chem.*, 163: 129–135, 1946.

Mason, K. E.: Differences in testis injury and repair after vitamin A-deficiency, vitamin E-deficiency, and inanition. *Am. J. Anat.*, 52: 153–239, 1933.

Mason, K. E., and E. T. Ellison: Changes in the vaginal ephithelium of the rat after vitamin A-deficiency. *J. Nutrition*, 9: 735–755, 1935.

Schurr, P. E., H. T. Thompson, L. M. Henderson, and C. A. Elvehjem: A method for the determination of free amino acids in rat organs and tissue. *J. Biol. Chem.*, 182: 29–37, 1950.

Schurr, P. E., H. T. Thompson, L. M. Henderson, J. N. Williams, and C. A. Elvehjem: The determination of free amino acids in rat tissues. *J. Biol. Chem.*, 182: 39–45, 1950.

Solomon, J. D., C. A. Johnson, A. L. Sheffner, and O. Bergeim: The determination of free and total amino acids in rat tissues. *J. Biol. Chem.*, 189: 629–635, 1951.

Stein, W. H., and S. Moore: Electrolytic desalting of amino acids—conversion of arginine to ornithine. *J. Biol. Chem.*, 190: 103–106, 1951.

Williams, R. J., and H. Kirby: Paper chromatography using capillary ascent. *Science*, 107: 481–483, 1948.

Wolbach, S. B., and P. R. Howe: Tissue changes following deprivation of fat-soluble A vitamin. *J. Exptl. Med.*, 42: 753–777, 1925.

AMINO ACID METABOLISM IN VITAMIN DEFICIENCY:

II. The Glutamic-Aspartic Transaminase Activity in Tissues from Normal and Deficient Rats

Mohammed Abdulnabi and J. G. Bieri*

The possible involvement of vitamin A in protein metabolism has been referred to in the preceding paper (Abdulnabi and Bieri, 1953). Since the transaminases are actively involved in protein and amino acid metabolism, one possible mechanism by which vitamin A might be involved in nitrogen metabolism is through the transamination reaction. Although no information is available in the literature concerning this possibility, it has been reported recently that the transaminase system might be related to a fat-soluble vitamin deficiency (Barber, Basinski, and Mattill, 1949). The present paper deals with the study of the activity of the glutamic-aspartic transaminase system in the kidney, liver, and heart of normal and vitamin A deficient rats. Of these tissues, only the kidney is known to be primarily affected in vitamin A deficiency (Wolbach and Howe, 1925; Tyson and Smith, 1929).

Experimental. The method used for the determination of the glutamic-aspartic transaminase activity was essentially the manometric procedure of Green, Leloir, and Nocito (1945). The preparation of animals for these determinations was similar to that reported in the previous paper (Abdulnabi and Bieri, 1953). The food consumption was equalized by giving restricted amounts of the U.S.P. vitamin A assay ration. When the first stage of vitamin A deficiency appeared in an experimental animal, both the depleted rat and its control were decapitated and the livers, kidneys, and hearts rapidly extirpated and immediately packed in finely cracked ice. After cooling, bits of fat and connective tissue were removed, the tissues blotted between filter paper, and rapidly weighed. A homogenate of the tissue was prepared in ice-cold 0.1 M. sodium phosphate buffer (pH 7.4). This homogenate was pipetted immediately into the pre-cooled reaction vessels. The dry weights of samples were determined by evaporation of 1.0 ml. homogenate to constant weight in an oven at 100° C.

---

* From the Department of Biochemistry and Nutrition, The University of Texas Medical Branch, Galveston. The material in this paper is from a thesis submitted to the Graduate School by Mohammed Abdulnabi in partial fulfillment of the requirements of the Doctor of Philosophy degree. This work was supported, in part, by the Williams-Waterman Fund of the Research Corporation. Received for publication January 30, 1953.

Flasks with a single side arm were used in the direct method of Warburg at 37° C. The components of the final reaction mixture were: main chamber: 0.9 ml. of 0.25 M. sodium phosphate buffer (pH 7.4), 1.0 ml. of 0.2 M. sodium aspartate, 0.1–0.3 ml. of tissue homogenate in 0.1 M. sodium phosphate buffer, and a sufficient quantity of this same buffer to yield a total volume of 3.2 ml.; side arm: 1.0 ml. of 0.1 M. sodium α-ketoglutarate; center cup: 0.5 ml. of aniline citrate reagent. Three levels of each tissue were run in duplicate. These were 0.1, 0.2, and 0.3 ml. of 5 per cent liver and 2.5 per cent heart and kidney homogenates. After eight minutes equilibration readings were recorded and the stop-cocks of the manometers closed. The α-ketoglutarate was added from the side arm and the reaction allowed to proceed for exactly ten minutes. At the end of this time, the reaction was stopped by adding aniline citrate from the center well. This was mixed carefully to insure complete decomposition of oxalacetic acid formed during the reaction. After ten minutes, when all of the carbon dioxide had been liberated, the readings of the manometers were recorded. The carbon dioxide produced per mg. of wet tissue of each sample was calculated after subtracting the carbon dioxide evolved from the blank flask. This flask contained all reactants, including homogenate, but the

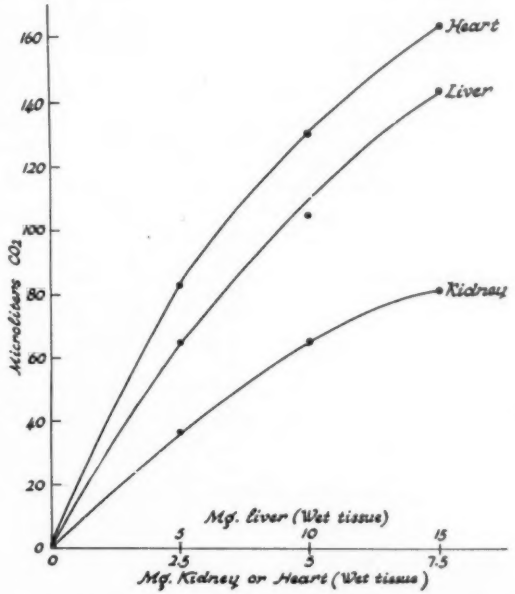


FIG. I. Representative curves of glutamic-aspartic transaminase activity of rat tissue homogenates.

aniline citrate reagent was added to the main chamber at zero time, thus stopping the enzymatic activity of the homogenates. The carbon dioxide liberated from the homogenates was found to be negligible compared with that liberated from the reagents.

Results and Discussion. Typical curves illustrating the aspartic-glutamic transaminase activity of the three tissues studied are shown in figure I. These curves clearly demonstrate that at the three levels of tissue used there is an essentially linear relationship between the amount of tissue and the observed enzyme activity. The values of carbon dioxide liberated by the three levels of tissue employed agreed, in general, within ±7 per cent for liver, ±15 per cent for kidney, and ±6 per cent for heart. A similar linearity with a purified preparation of this enzyme was shown by Green, Leloir, and Nocito (1945).

Table 1 is a summary of the results obtained from liver, kidney, and heart homogenates expressed on the basis of wet weight of tissue. Values for liver, on a dry weight basis, are also included to demonstrate that the comparison between normal and deficient animals is not altered by considering the tissue

TABLE 1

Transaminase Activity of Tissues of Normal and Vitamin A Deficient Rats. Expressed as Microliters Carbon Dioxide Liberated in 10 Minutes by 1 mg. Tissue

	Liver		Kidney	Heart
Normal Rats	Wet	Dry	Wet	Wet
1	8.7	30.4	10.6	20.7
2	10.9	39.8	9.8	23.8
3	11.7	45.0	8.7	23.2
4	13.5	39.9	12.8	23.1
5	12.2	49.2	10.1	22.0
6	12.2	47.3	12.0	26.5
7	6.3	24.2	3.7	19.9
8	11.0	50.5	5.9	19.7
9	10.9	53.4	10.4	22.4
Average ± S.D.	10.8±2.1	42.2±9.1	9.3±2.8	22.4±1.6
Deficient Rats				
10	12.1	40.1	10.6	22.2
11	14.8	54.4	12.7	26.0
12	7.3	25.5	8.1	20.3
13	10.3	38.7	8.2	20.7
14	17.1	75.0	6.3	26.5
15	9.1	31.8	9.5	23.3
16	11.6	48.7	5.6	22.0
17	10.9	36.8	8.8	23.8
Average ± S.D.	11.6±3.1	43.9±8.4	8.7±2.2	23.1±2.1

water content. A similar result was obtained from several kidney and heart samples, consequently, the dry weight values of these tissues were not included.

Of the three tissues studied, the heart was the most active, being about 100 per cent greater than liver, and about 140 per cent greater than kidney expressed on a wet weight basis. The activity of kidney was about 80 per cent that of liver. The values for heart show the least variation from the mean. With liver and kidney, although the variation is greater, the majority of values are grouped closely with only one or two values showing considerable deviation. As to the effect of vitamin A on the transamination reaction studied, there seems to be no difference in the activity of the enzyme in tissues from normal or deficient animals. This indicates that vitamin A deficiency does not affect the glutamic-aspartic transaminase activity.

Summary

The glutamic-aspartic transaminase activity has been studied in homogenates of liver, kidney, and heart from normal and vitamin A deficient rats. Using a manometric method, no differences between normal and vitamin A deficient animals were found. Of the three tissues investigated, the heart was the most active, while the activity of kidney was slightly lower than that of liver.

REFERENCES

Abdulnabi, M., and J. G. Bieri: Amino acid metabolism in vitamin A deficiency. *Tex. Rep. Biol. and Med.*, 10: 181–195, 1953.

Barber, M. A., D. H. Basinski, and H. A. Mattill: Transamination in the muscles of animals deprived of vitamin E. *J. Biol. Chem.*, 181: 17–21, 1949.

Green, D. E., L. F. Leloir, and V. Nocito: Transaminases. *J. Biol. Chem.*, 161: 559–582, 1945.

Tyson, M. D., and A. H. Smith: Tissue changes associated with vitamin A deficiency in the rat. *Am. J. Path.*, 5: 57–69, 1929.

Wolbach, S. B., and P. R. Howe: Tissue changes following deprivation of fat-soluble A vitamin. *J. Exptl. Med.*, 42: 753–777, 1925.

APPARATUS FOR STABILIZING THE CHEMICAL AND PHYSICAL ENVIRONMENT OF CERTAIN FREE-LIVING CELLS*

IBEN BROWNING† AND LLOYD S. LOCKINGEN

The purpose of this paper is to describe a new device for controlling the rate of growth and population density of cultures of the protozoan *Tetrahymena geleii*, and to suggest its use in studies under sterile conditions for these and other kinds of free-living cells.

Novick and Szilard (1950) developed a device termed a Chemostat for keeping a bacterial population growing at a reduced rate. Several attempts to use a Chemostat of this design for controlling growth rates of *Tetrahymena geleii* were unsuccessful due to the nature of the medium used in culturing this organism. There are two primary reasons: (1) bubbling of medium in the storage reservoir produced excessive foaming and intermittent stoppage of the capillary leak; (2) adhesion of medium components to the inner walls of the metering tip of the delivery tube caused a progressive reduction in flow rates.

The sintered glass filter in the delivery tube of the Chemostat was not used because of the demonstrated effect of medium filtration in the case of the protozoan *Paramecium multinucleata* (1951) and evidence at hand in this laboratory tentatively suggests similar effects of filtration of medium on the growth characteristics of *Tetrahymena geleii*.

Construction. The assembly consists of three distinct units: a replaceable medium reservoir, a pump unit, and a growth flask.

Medium Reservoir. The medium reservoir is a standard Pyrex Erlenmeyer flask, equipped with the three tubes installed through a paraffin sealed rubber stopper. These tubes may be identified in figure I as follows: (A) This tube delivers medium

* Department of Biology, The University of Texas, The M. D. Anderson Hospital for Cancer Research, Houston. This paper owes its completion to the unceasing efforts of Mary Walters, Janet Bergendahl, Madeleine Brittain, James McNeese, and Norma Martin. Received for publication February 10, 1953.

† Present address: Development Department, Instrument Division, American Optical Co., Buffalo 15, New York.

to the pump unit; (B) This tube communicates with the outside atmosphere and thereby establishes the hydrostatic head under which the system operates and consequently the level to which the medium will rise in the input reservoir of the pump unit; (C) The purpose of this tube is solely for starting the siphoning action when a fresh flask of medium is installed. It is clamped off after siphoning has started; this system is based on the same principle as that of the standard Mariotte bottle.

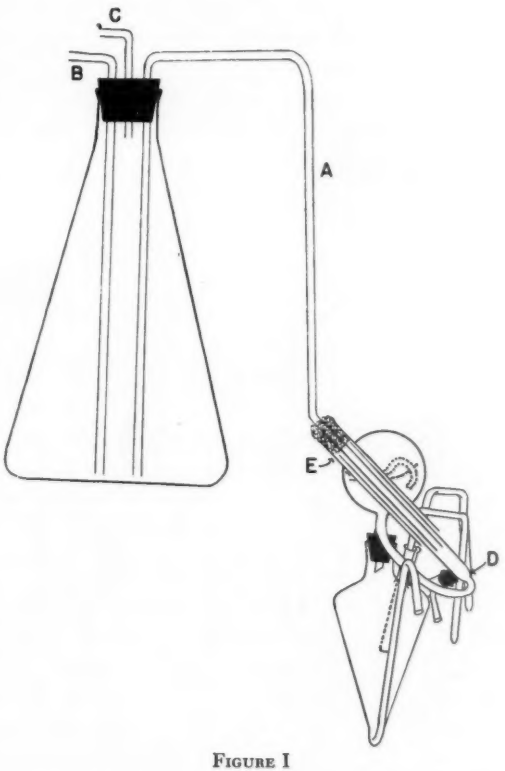


FIGURE I

Pump Unit. The shell for this unit was fabricated from a standard 125 ml. Pyrex Erlenmeyer flask. An indentation made

in the side provides a reservoir when the flask is in operating position. Other features are identified as follows in figure II: (D) This large tube receives the delivery tube (A) from the medium reservoir; (E) This tube is used to maintain sterility at the junction of tubes (A) and (D) (See figure I for assembly details); (F) This semi-circular dipper (see figure III for details) was made by splitting a 6 mm. Pyrex tube longitudinally and then forming it about a central drive shaft similar to an Archimedes Screw. The drive shaft passes through a Silicone packed tube fixed in a paraffin sealed rubber stopper. The driving mechanism is attached to the end of this shaft and, in the case of the model illustrated in figure V, a 4 R.P.M. 4 watt synchronous motor is the power source; (G) Medium is delivered to the growth flask through this tube.

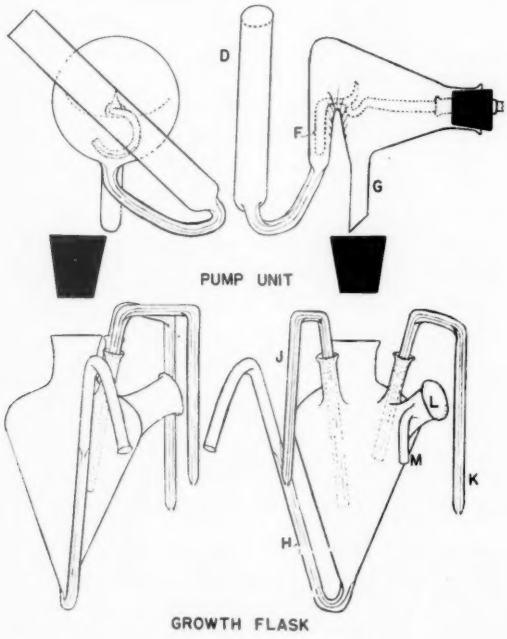


FIGURE II

Growth Flask. Several different flasks have been used with this apparatus. The flask illustrated was used in a special experiment to determine whether or not a differential existed in growth rates in the capillary tube and in the flask. The essential characteristics of this flask are as follows: (H) This capillary tube is used for aerating the culture; (J) This capillary tube is a constant level siphon; it may be adjusted to establish a given operating liquid volume in the flask; (K) This capillary tube is peculiar to this flask and was used to remove large samples. It may be used for a similar purpose in other experiments; (L) The large opening is used for inoculation; (M) This is a gas escape port.

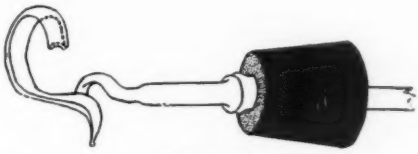


FIGURE III

All ports and air lines, except the inoculation port are cotton filled to maintain sterility during autoclaving. The latter is closed with a rubber stopper, and is capped with aluminum foil when not in use. The movable siphons (J) and (K) are sealed with Silicone stopcock grease.

Operation. Prior to commencing operation, the parts for one or more medium flasks are assembled, the flask is filled to about 2/3 capacity with medium and autoclaved. The pump and growth flask are assembled as a single unit and are autoclaved at the start of each new experiment. The pump and growth flask are installed in a cabinet as illustrated in figure V. Cotton plugs used in autoclaving are removed, tubes (D) and (E) are flamed and the medium reservoir is installed in place. Siphoning action is started by applying pressure through tube (C). As soon as siphoning starts, this tube is clamped off. The medium level in the pump reservoir is adjusted by moving the pump and flask along an inclined plane.

The speed of the motor then governs the rate of flow of medium into the growth flask. Adjustment of the constant level siphon (J) is made to establish the culture volume desired.

Thermostatic control is necessary for controlled growth of *Tetrahymena geleii*. This was attained at first by mounting the three basic units in a standard paraffin oven. The necessity for portability suggested the construction of the box illustrated.

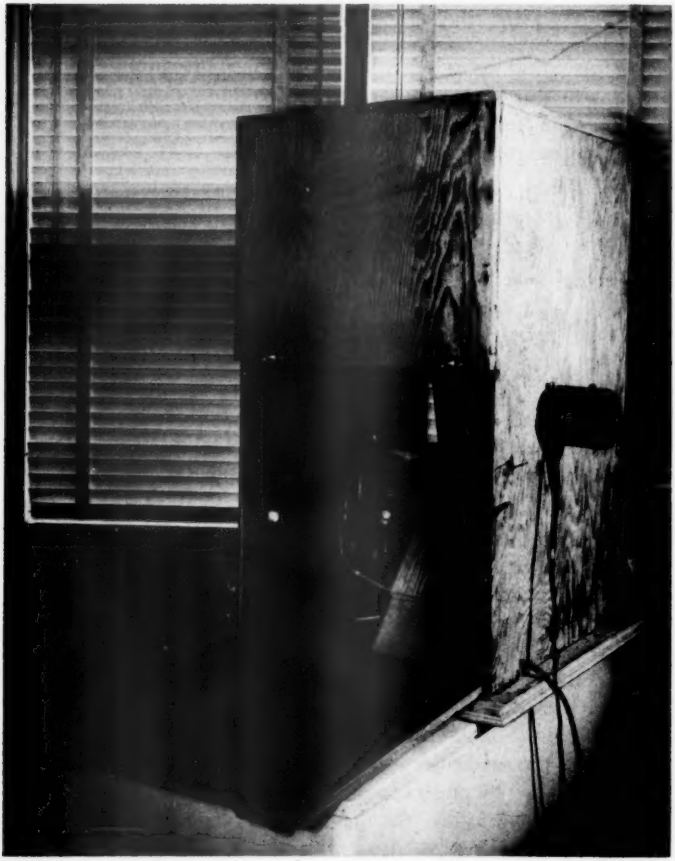


FIGURE V

Unique Characteristics. 1. The use of relatively large bore tubing and the design of the pump unit permit the delivery of a

finely particulate homogeneous medium without change of delivery rate.

2. The design of the dipper is such that temperature and barometric pressure changes which have a small effect on the hydrostatic head do not change the rate of feed into the growth flask. Over a considerable range, the volume of liquid delivered by the dipper is independent of the depth of penetration into the liquid.

3. The device has proved satisfactory for certain studies with *Tetrahymena geleii* and appears to be adaptable for use with other kinds of free-living cells requiring similar conditions.

4. The use of a roller and disc drive as illustrated in figure IV permits a wide range of medium delivery rates with a very low minimum rate. A model using this principle has been used in this laboratory.

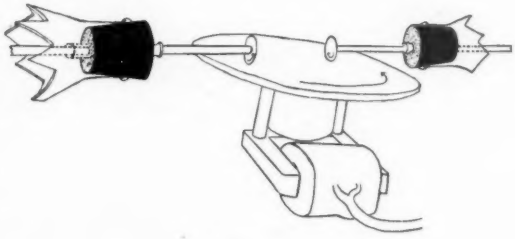


FIGURE IV

5. It is possible to operate this apparatus either at a constant rate or intermittently dependent upon some criteria such as optical density or pH. For example, pH meter electrodes could be installed in the growth flask and, with modified wiring, could be used to trip a relay and consequently to start or stop the flow of medium within a preset pH range.

Suggested Uses. As pointed out by Novick and Szilard the principal purpose of machines of this type is to hold a culture at an equilibrium population. This equilibrium population is dependent on three factors: The rate of flow of medium into the growth flask, the volume of the culture, and the growth factor content of the input medium. By varying these factors it is possible to obtain an equilibrium population at any reproduction

time greater than the minimum generation time of the organisms under the imposed conditions.

It would be possible to drive two pumps with independent rollers from a single motor driven disc (see Fig. IV), such that regardless of relative rates, a constant sum-total rate of feed results. Two such pumps could be used to supply a single growth flask in such a way as to measure the effect of a test-chemical or perform a microbiological assay using reproduction or some other criterion as a measure of effect. Using recording equipment, this sort of design could lead logically to a machine which would automatically perform an entire microbiological assay.

This device is utilizable for growing populations under conditions approaching constancy for indefinite thousands of generations, and should prove ideal for the population geneticist who might want to study evolution in a constant radiation field; select for special kinds of mutants; etc.

The ease of adjustability from a stable population of short-lived to a stable population of long-lived cells should make the device attractive to physiologists and geneticists interested in the problems associated with age.

Summary

A device for controlling the environment of organisms has been described.[1] Several suggestions have been made regarding its potential usefulness as a biological research tool.

REFERENCES

Novick, Aaron, Leo Szilard: *Science*, 112: 2920, 1950.
Rudzinska, Maria: *Proc. Soc. Exptl. Biol. and Med.*, 78: 1, 1951.

[1] Manufacture of this device is being planned.

THE ALTERATION OF PLASMA AND AORTAL LIPIDS BY ADENOSINE-5-MONOPHOSPHATE*

WILLIAM W. CALHOUN, LAWRENCE J. MILCH, ROBERT F. REDMOND, AND THE CARDIOVASCULAR RESEARCH GROUP†

A previous report from this laboratory (Milch, *et. al.*, 1952) indicated that administration of adenosine-5-monophosphate (AMP)[1] to atherosclerotic hens induced a marked diminution of aortal cholesterol concentration. Similar results were observed with young cockerels in which aortal lipid concentrations had been previously elevated by a high cholesterol diet. There remained to establish the effect of AMP on the apparently normal aorta—unaltered by either an unphysiological diet or extensive atherosclerotic pathology.

Rodbard, *et al.* (1952), have shown that between the ages of 4 and 6 months there is a regression of atherosclerosis in young cholesterol-fed cockerels. It is during this period that relatively small amounts of cholesterol could reasonably be expected in normal cockerel aortæ, and total plasma cholesterol values would be correspondingly minimal.

It was further noted (Milch, *et al.*, 1952) that control was exercised, in the experiments involving AMP-treated hens and cockerels, by intramuscular injections of physiological saline to parallel groups of fowl. Inasmuch as the AMP is actually supplied by the manufacturer as a gelatin suspension, such saline control may not have been adequate.

Finally, it was reported (Milch, *et al.*, 1952) that S_f 12–20 lipoprotein concentrations in atherosclerotic hens may have actually increased after seven days of AMP administration. Such behavior seemed peculiar in view of the trend toward lower values of total serum cholesterol concentration.

It was felt that a more prolonged period of AMP administration, using gelatin for control injections and further using cockerels at 5 months of age, might clarify these points.

---

* From the Department of Pharmacology and Biochemistry, USAF School of Aviation Medicine, Randolph Field, Texas.

† Clarence W. Johnson, Richard A. Yarnell, William H. Graves, Jr., John G. Collins, James V. Stinson, and Normal L. Baxter.

[1] "My-B-Den," Ernst Bischoff Co., Ivoryton, Conn.

Experimental. Sixty 5-month-old, single comb White Leghorn cockerels were placed in cages in our laboratory and maintained on standard commercial ration for 2 weeks prior to the experiment. At the beginning of the experimental period, the animals were divided into 2 equal groups. One group was injected intramuscularly with 55 mg. of AMP each day for 3 days and thereafter every other day for 10 days (8 injections). The control group was similarly injected on the same days with gelatin placebo. On the fifteenth day, the animals were bled by heart puncture with a lightly heparinized syringe, sacrificed, and the aortæ excised from arch to abdominal bifurcation. The aortæ were carefully separated from adhering fat and connective tissue and examined grossly for any signs of atherosclerotic lesions. No evidence of atherosclerosis was observed in either group.

The blood was centrifuged to yield unhemolyzed plasma. These plasma samples were analyzed for total cholesterol and the S_f 12–20 and S_f 20–100 classes of lipoproteins. The aortæ were placed in fat-free thimbles and dried to a constant weight under an infrared lamp. The dried aortal tissue was analyzed for cholesterol concentration. The details of the analytical procedures as done in this laboratory have been previously described (Redmond, *et al.*, 1952).

The Identity of Plasma and Serum Lipoprotein Analyses. It should be noted that the data reported herein were obtained on *plasma.* Data on the 7-day administration of AMP previously reported (Milch, *et al.*, 1952) were taken on serum. In order to establish the fact that lipoprotein analyses were identical whether done on plasma or serum, 2 rabbits[2] were bled successively, each with an unheparinized and then with a heparinized syringe. The samples were separately subjected to ultracentrifugal lipoprotein analysis. Two of the resulting photographic traces are shown in figure I. The upper plate contains the plasma and serum analyses from rabbit A and the lower plate contains similar analyses on the two samples from rabbit

[2] Adequate blood for duplicate analyses on serum and plasma could not be obtained from chickens since it is extremely difficult to insert a second needle in the heart after one sample has been taken. Therefore, rabbits were used to establish this comparison.

B. The identity of results within each individual is readily established by inspection.

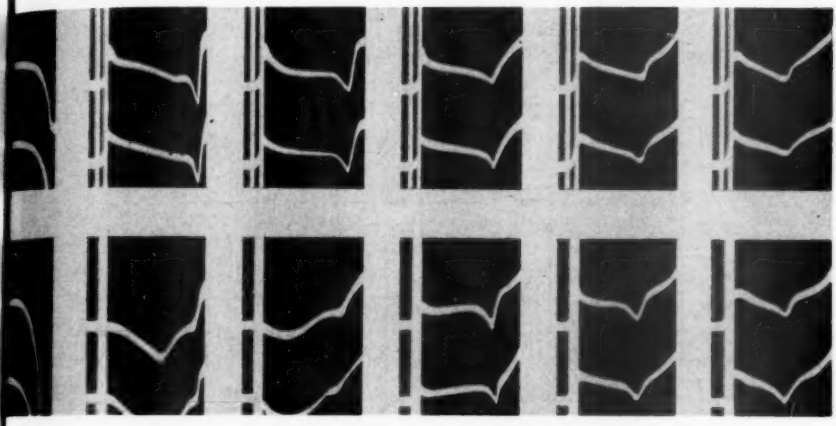


FIG. I. Comparison of Ultracentrifugal Analysis of Lipoproteins on Serum and Plasma. The Serum Pattern is Shown Above the Corresponding Plasma Sample in Each of the Above Examples.

Discussion. The data indicate that AMP has the same effect on apparently normal chicken aortæ as was observed previously in atherosclerotic hens and in cholesterol-fed cockerels. According to Table I, intra-muscular administration of AMP, for 14 days, induced a significant decrease in aortal cholesterol concentration (from a control level of 6.1 mg./gm. to 4.5 mg./gm.). By way of confirmation, it should be noted that the value of 6.1 mg./gm. recorded for the present control group, is in good agreement with the average of 6.95 mg./gm. previously reported for control cockerels at an age when slightly more cholesterol would be expected (Milch, *et al.*, 1952; Rodbard, *et al.*, 1952). There would seem to be little doubt that exogenous AMP exercises a marked influence on aortal lipids, regardless of source. Furthermore, these data demonstrate that the gelatin carrier is not involved in the observed effect of AMP.

It would seem that the net effect of a more extended period of AMP administration on blood plasma cholesterol concentration is not unlike the previously reported results obtained by one week of treatment. The mean of the plasma total choles-

terol values for this group of control animals is 188 mg. per cent. AMP administration resulted in a significant drop in plasma cholesterol to a mean value of 163 mg. per cent (P=0.01). Neither cholesterol feeding, nor the diseased (atherosclerotic) state was found to be necessary for this *in vivo* effect of AMP.

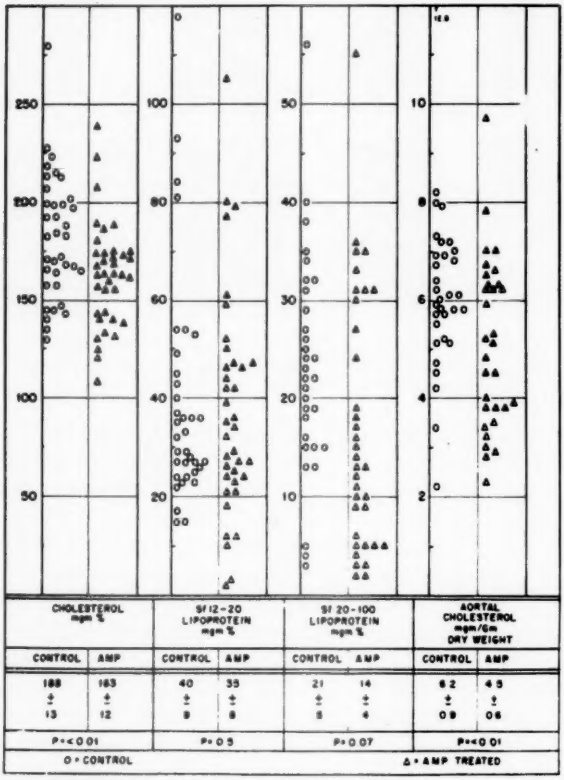


TABLE 1

The Results of Cholesterol and Lipoprotein Analyses of Control and AMP-Treated 5-Month-Old Cockerels. Mean Values Are Given ± Twice the Standard Error of the Mean.

The values herein reported for the two classes of plasma lipoproteins may be revealing. Fourteen days of AMP administra-

tion failed to alter the S_f 12–20 class (P=0.5). On the other hand, a tendency toward a decrease of S_f 20–100 is recorded (P=0.07). No adequate explanation of these findings is possible at this time. It should, however, be remembered that the data reported for atherosclerotic hens showed a tendency (P=0.07) for an increase in S_f 12–20 lipoproteins as a result of AMP administration for a period of one week.

The difficulty of interpretation of these findings is best demonstrated by a brief review of our present knowledge concerning lipoprotein metabolism. Gofman, *et al.* (1952), have shown that the normal occurrence is a successive conversion of low density high S_f lipoproteins toward higher density lower S_f value molecules and eventual catabolism. These same authors feel that a pile up of S_f 12–20 and S_f 20–100 classes of lipoproteins in rabbits and men results in an increased atherogenic tendency, and are seeking means of altering the concentration of these two classes of lipoproteins in blood serum or plasma. Thus far they have shown (Graham, *et al.*, 1952) that heparin seems to increase the rate of conversion to lower S_f classes of molecules, but no changes in the total serum or plasma cholesterol have been reported as a result of heparin administration. In view of the absence of marked reorientation of cholesterol-bearing lipoproteins, but the significant decrease of both plasma and aortal concentration of total cholesterol, it would seem that AMP exerts an effect which is different from that of heparin.

Summary

AMP was administered to 5-month-old, non-atherosclerotic cockerels over a 14-day period and found to be associated with significantly lowered plasma and aortal cholesterol concentrations. No significant change was recorded for either the S_f 12–20 or S_f 20–100 classes of lipoproteins as assayed ultracentrifugally. The gelatin carrier in which the AMP is suspended was demonstrated not to participate in the AMP effect. The role of AMP in lipoprotein metabolism discussed.

REFERENCES

Gofman, J. W., personal communication.

Graham, D. M., T. P. Lyon, J. W. Gofman, H. B. Jones, A. Yankley, and J. Simonton: The Influence of Heparin on Lipoprotein Metabolism and Atherosclerosis. *Circulation*, 4: 465, 1951.

Milch, L. J., R. F. Redmond, W. W. Calhoun, H. I. Chinn, and the Cardiovascular Research Group: Effects of Adenosine-5-Monophosphate on Serum and Aortal Lipids in the Chicken. *Am. J. Physiol.*, 170: 346–350, 1952.

Redmond, R. F., L. J. Milch, W. W. Calhoun, H. I. Chinn, and the Cardiovascular Research Group: The Evaluation of Clinical Tests for Atherosclerosis. I. Instrumentation. *Project No. 21-32-027, Report No. 1*, August, 1952, USAF School of Aviation Medicine, Randolph Field, Texas.

Rodbard, S., R. Pick, C. Bolene-Williams, L. N. Katz: Age-Conditioned Spontaneous Regression of Atherosclerosis in the Cholesterol-fed Chick. *Circulation*, 6: 459–260, 1952.

THE OCULAR CHANGES IN DIABETES AND THEIR EFFECT ON THE PATIENT'S VISION*

FREDERICK C. CORDES

It is a great honor to have been asked to deliver the John O. McReynolds Lecture. John O. McReynolds was one of a group of pioneer American ophthalmologists who laid the ground work for the present high standard of ophthalmology in this country. This group of men included such famous names in ophthalmology as John E. Weeks, Charles May, and George DeSchweneinitz. Dr. McReynolds early acquired a skill in ophthalmic surgery which became widely known an dbrought him patients from all over the southwestern United States and Mexico. His contributions to ophthalmology were many but his best known contribution to ophthalmic surgery was the transplantation of pterygia into the lower fornix, a procedure still practiced by most American ophthalmologists and known the world over by his name. He was also one of the early intracapsular operators for cataract surgery. The many honors conferred upon him are tribute to his genius.

It was my privilege to become acquainted with Dr. McReynolds in 1922 when he attended the A.M.A. meeting in San Francisco. At a dinner given in honor of Dr. DeSchweinitz at the Bohemian Club it was my privilege to sit next to Dr. McReynolds and to hear from him some of his early experiences in Texas, an evening I shall not forget.

It is always pertinent to discuss the ocular complications of diabetes and the role they play in determining the patient's visual prognosis. Changes in the course of diabetes since the introduction of insulin have made it necessary for the ophthalmologist to revise the former concepts of the effect the disease may have on the patient's vision. Insulin therapy has prolonged the expectation of life in the diabetic and has led to an increasing proportion of diabetics with degenerative retinal changes.

---

* The John O. McReynolds Lecture, delivered at The University of Texas Medical Branch, Galveston, March 31, 1953. From the Department of Ophthalmology, and the E. S. Heller Laboratory of Eye Pathology, University of California Medical Center, San Francisco.

Wagener in 1921 found that approximately 8 per cent of diabetics at the Mayo Clinic revealed diabetic retinopathy; in 1945 he found that 29.6 per cent of diabetics had retinopathy. In addition those who had had the disease less than 15 years the incidence was less than 11 per cent. In the group who had had the disease for more than 20 years the incidence was 73 per cent. Thus it is apparent that insulin, in prolonging the life expectancy of the diabetic, has increased the percentage of diabetics with retinopathy. This applies particularly to the juvenile diabetic who now for the first time is being observed by the ophthalmologist.

Diabetes is a metabolic disease in which there are symptoms of polyuria, polydipsea, voracious appetite, progressive loss of flesh and strength and a tendency to fatal termination. The disease is essentially due to an upset of the balance between insulin and its antagonists; the latter consisting of hormones produced by the pituitary, thyroid and adrenal cortex. As a rule absolute as well as relative deficiency are found, but relative deficiency is the decisive factor.

There are two recognized types of diabetics: the hyperfunctional type in which the pancreas is unable to deal with the hyperproduction of sugar caused by hypophyseal stimulation; and the rare hypofunctional type which is primarily pancreatic disease. Appel states that 70 per cent of diabetics belong to the hyperfunctional type; of this group 80 per cent also show hypertension. In the hypofunctional type hypertension is no more frequent than would be expected in their relative age groups.

Diabetes mellitus is more frequent in women than in men and in most instances is diagnosed between the ages of 40 and 70. Heredity is a definite factor and the tendency to diabetes is probably transmitted as a simple Mendelian recessive.

There seems to be a racial difference in susceptibility, the French for example, being less liable to diabetes than other countries. Many authorities, however, believe that the racial incidence of diabetes is not entirely attributable to race as such but rather to the dietary habits of the race, some races being prone to overeating. There seems to be little doubt that obesity is a definite predisposing factor. Two specific manifestations of diabetes are Kimmelstiel-Wilson disease (intercapillary glomerulosclerosis) and retinopathy.

General Prognosis. A review of the literature would indicate that at the present time diabetes does not diminish the average expectation of life. However, statistics of life insurance companies stress the menace of obesity.

In addition to ocular complications certain other complications are found. Martenson in a follow-up of 221 diabetics who had survived at least 15 years, found that 57 per cent had arterial hypertension; 43 per cent had coronary arteriosclerosis, and 34 per cent had renal disease. Engel in

studying 644 cases found that 25 per cent under 49 years of age had renal disease.

In some cases where the diagnosis of diabetes has been a factor in the patient controlling his weight and exercising greater care in his well-being, diabetes may even be considered a factor in the prolongation of life. However, diabetes contracted in childhood or adolescence "loads the scales against longevity."

Ocular Complications. There are a number of ocular complications that are the result of diabetes. *Wrinkling of the cornea and weakness of accommodation* are two conditions that occur in about 20–25 per cent of diabetics. These conditions clear when the disease is brought under control. A change in the refractive error has been observed many times in diabetes.

There is a *myopia*, or nearsightedness, associated with a rise and *hyperopia*, for farsightedness, with a fall in the sugar concentration of the blood. The hyperopic change seems not to occur as an initial phenomenon but to follow a myopic change. With a clinical variation of the blood sugar concentration the refractive state of the eye may follow changes from hyperopia to myopia and vice versa. This alteration of refraction is frequently associated with astigmatic changes. In contrast to the rapid development of hyperopia after the myopia the regression to a normal state is gradual, generally requiring two to four weeks.

Since Elschnig reported a case that had been operated upon for cataract in one eye in which the refractive changes occurred only in the unoperated eye it has been generally accepted that the changes in the lens are the cause of the refractive changes.

Duke-Elder explains the transitory myopia as attributable to fluid entering the lens due to osmosis resulting from a variation in the sugar concentration of the blood and the tissue fluids. Whatever the cause of the changes may be, a sudden and unexplained myopia should always suggest the possibility of diabetes and make the ophthalmologist insist on at least a urine examination. It is surprising how often this symptom may be the first to call the disease to the patient's attention. If it occurs in a known diabetic an immediate check-up is indicated to be sure there has not been a relapse.

A sudden hyperopic change may indicate treatment being too suddenly undertaken or too rigorously applied. This has been seen much more frequently since the introduction of insulin.

The treatment of these transient refractive changes should be directed toward the constitutional disease. Since the refractive state of the diabetic may require two to four weeks to be stabilized, glasses should not be prescribed until this period has elapsed. The patient should also be warned about future possible sudden changes and their significance.

Aneurysms of the Conjunctiva. In 1950 McCulloch and Pashby in examining 100 diabetics and 50 normals found conjunctival aneurysms in 55 per cent of the diabetics and 14 per cent of the normals. Two types were seen; the first type was a berry-like aneurysm of the type described by Ballantyne as occurring in the retina. These aneurysms were purplish in color as distinct from small hemorrhages. These aneurysms do not absorb and can be seen unchanged as to size, shape and position over several months.

The second type was seen as an aneurysmal dilatation of the veins; was seen anywhere in the conjunctiva but usually near an area of inflammation. This type is very common and has no possible bearing on diabetes.

Conjunctival aneurysms of the berry type were four times as common in diabetics as in normal individuals and were found in 62 per cent of the cases of diabetic retinopathy so that while these conjunctival aneurysms are not limited to diabetics they are rather suggestive of the possibility of the disease.

Iris Changes. There are two types of lesion of the iris seen in diabetes. *Iridopathy* is an extreme condition of edema of the pigment layer with formation of numerous small cystic spaces common in diabetes. This is due to the pigment epithelium being filled with glycogen. As a result of the formation of these cysts and blisters the pigment is liberated and may at times be seen as pigment spots on the anterior lens surface. The freed pigment in the anterior chamber is seen at times when the anterior chamber is opened during a cataract operation. *Rubeosis Iridis Diabetica* is used to describe the presence of new formed blood vessels in the sphincter region of the anterior surface of the iris in diabetes. Microscopically it consists of a fibrovascular membrane on the iris surface. Glaucoma is always present and recurrent hyphemia is characteristic. Retinopathy is usually present in these cases. The prognosis is always bad in rubeosis

iridis as these eyes do not respond to miotics and antiglaucomatous surgery. If cyclodiathermy does not control the pain enucleation is the only recourse.

Cataract Formation in Diabetics. There are two types of cataract that may occur in the diabetic: (1) the true diabetic cataract which is seen only rarely, and (2) lens changes similar to the senile cataract of elderly non-diabetics.

(1) *True diabetic cataract.* Typical diabetic cataracts, invariably bilateral, are usually seen in young individuals with severe diabetes. O'Brien and Allen found them in 14 per cent of 260 diabetics under 21 years of age. They develop in the presence of a high concentration of sugar and usually when the patient is fairly well, *i.e.*, when he has sufficient reserive fluid. They usually appear in patients under 25 years of age and have been seen as early as 11 months. On rare occasions they occur

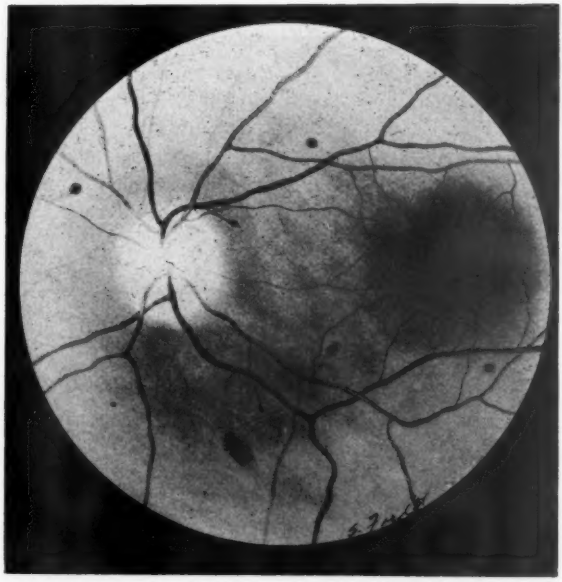


FIG. I. Diabetic retinopathy, Stage 1 (H. P. Wagener, T. J. Dry, and R. M. Wilder: Retinitis in Diabetes, *New England J. Med.*, 211: 1131–1137, Dec. 20, 1934) presenting punctate hemorrhages and capillary aneurysms. All the hemorrhages are essentially round.

in older individuals. The appearance of these true diabetic cataracts is characteristic. The opacities are of the "snow-flake type," start subcapsularly and increase rapidly. In addition to the typical lesions, irregular large and small placques may appear with fine, dust-like, dirty brown opacities, and rapidly progressive, bluish-white opacities.

(2) *Senile cataract in diabetes.* While not all authorities concur, it is generally agreed that the senile type of cataract appears more frequently in diabetic than in non-diabetics and at a relatively early age. The severity of the disease has no apparent relation to the incidence of the cataracts. The incidence increases with the duration of the disease.

Diabetic Retinopathy. It has been shown that there is no close relationship between the development of retinopathy and the severity of the disease. While arteriosclerosis and hypertension are often associated with diabetic retinopathy they are separate

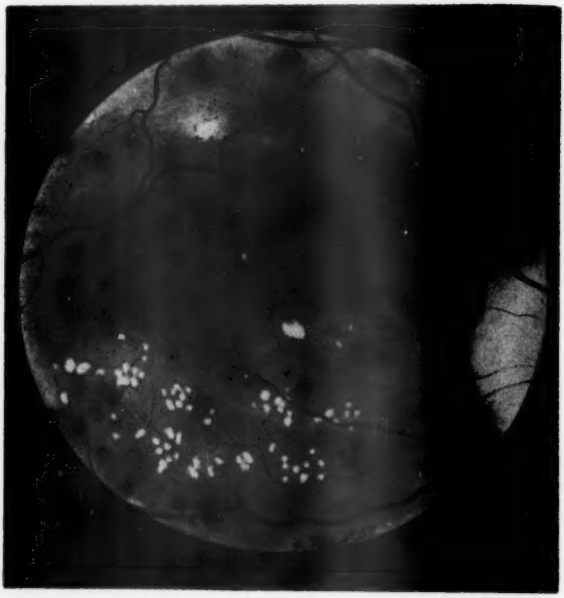


FIG. II. Diabetic retinopathy, Stage 2, representing the "central punctate retinitis" of Hirschberg (enlarged tracing of fundus photograph).

entities. The earliest recognizable lesions in both are pathological changes in the retinal vessels; diabetes involving primarily the venous system, hypertension primarily the arterial system. As evidence that hypertension and arteriosclerosis are not necessarily associated with diabetic retinopathy, diabetic retinopathy has been reported a good many times in the younger age group where there is no evidence of arterial disease. The duration of the disease is the most important factor in the production of the retinopathy.

Wagener divides the development of the retinopathy into five stages as follows: (1) The earliest sign is the appearance of one or more small capillary aneurysms which formerly were called "small deep hemorrhages." These were first demonstrated by Ballantyne and Lowenstein and later by Friedenwald, Ashton and others. Next there are tiny punctate hemorrhages that appear in the macular area (Fig. I).

FIG. III. Late Stage 2, with exudate coalesced into larger masses (enlarged tracing of fundus photograph).

(2) In stage 2 the exudates begin to appear. They are in the central area, soapy or waxy in appearance, well defined and yellowish in color. These small punctate exudates are referred to as the central punctate retinites of Hirshberg (Fig. II). At a later stage they have been described as looking like "blobs of white or yellow paint" (Fig. III). Distributed irregularly in the central area, they frequently form a circle around the macula (Fig. IV). As time goes on these exudates tend to coalesce into lardaceous irregular masses interspersed with dark grey pigmentary degeneration.

Duke-Elder in a personal communication stated that he and Ashton feel that there is a relationship between the fatty, or hard exudates and the microaneurysms. They feel the lipoids leak out through the wall of the aneurysms due to the fact that the aneurysms do not contain endothelium. Therefore these aneurysms may be surrounded by a circinate form of this exudate.

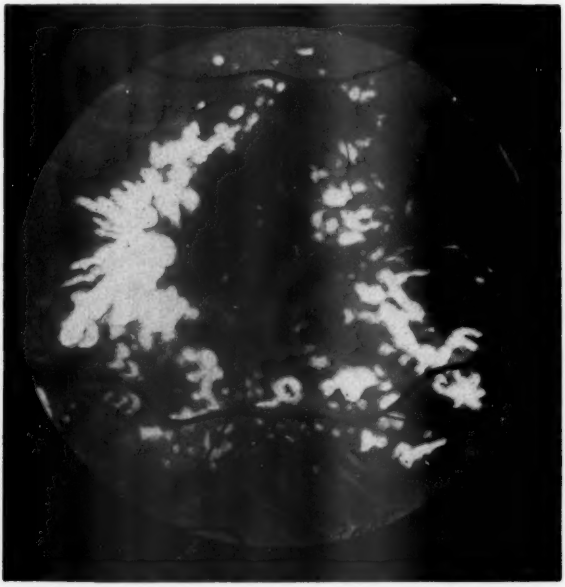


FIG. IV. Diabetic retinopathy, Stage 2, circinoid form (enlarged form tracing of fundus photograph).

They are of the opinion that exactly the same process takes place in the kidney in Kimmelstiel-Wilson's Disease.

(3) In this stage is added the presence of cotton-wool patches which may indicate the onset of complicating hypertension (Fig. V).

(4) In stage 4 visable changes occur in the veins; they became dilated, develop dilations (beadings) and become ensheathed (Fig. VI). In the young diabetic the fourth stage may be the first step in the development of the retinopathy. During this stage multiple thrombosis may develop and there may even be a total obstruction of the central retinal vein. There are also not infrequently coils and loops that develop in the veins of the periphery. These changes in the veins (beading, irregular expansion and contraction, coils and loops) are found only in diebetes.

(5) In the final stage there are recurrent vitreous hemorrhages. These are followed by retinitis proliferans which usually

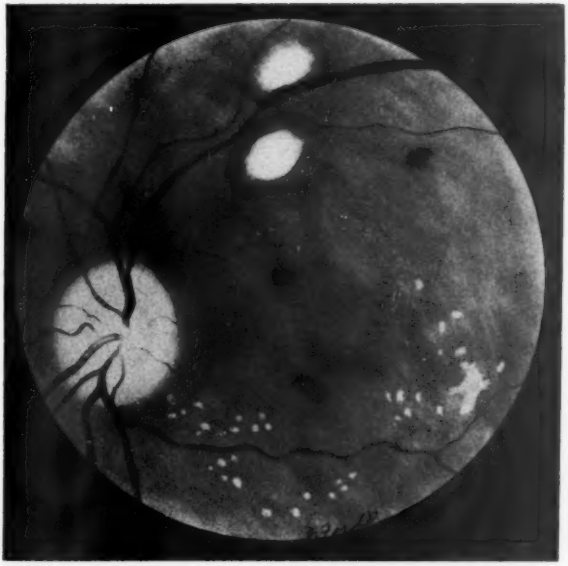


FIG. V. Diabetic retinopathy, Stage 3, showing "central punctate retinitis" with cotton-wool patches (enlarged tracing of fundus photograph).

leads to detachment of the retina and blindness (Fig. VII). This retinitis proliferans consists of proliferation of connective tissue from the disc, or nearby, and the development of a profusion of newly formed blood vessels.

Kimmelstiel-Wilson Disease. While diabetes affects primarily the venous side, there are a group of cases in which diabetes mellitus, albuminurian and hypertension are associated, the so-called Kimmelstiel-Wilson disease or intercapillary glomerulosclerosis. The lesion is characterized by hyaline changes in and around the glomerular tufts and capillaries associated with enlarged and dilated capillaries in the glomeruli.

The fundus lesion in addition to the diabetic changes shows changes of hypertension, cotton-wool patches and edema. Friedenwald, Ashton, Duke-Elder, and others feel that there is a close correlation between retinal microaneurysms and Kimmelsteil-Wilson's disease. Becker has pointed out that the renal

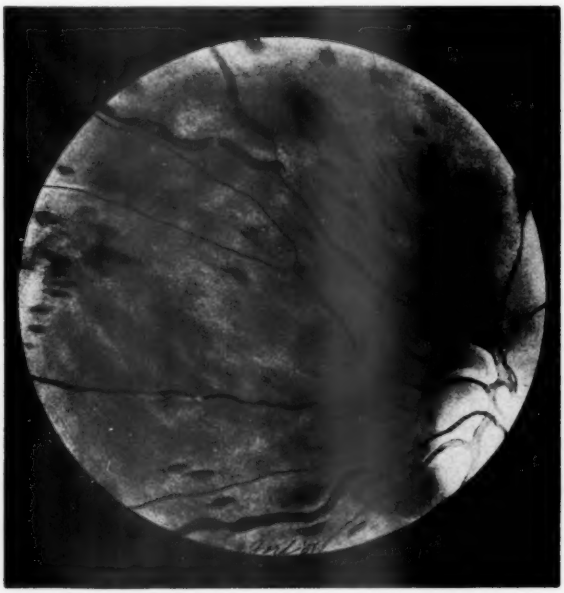


FIG. VI. Diabetic retinopathy, Stage 4, showing hemorrhages with pronounced disease of the veins (enlarged tracing of fundus photograph).

lesions of Kimmelstiel-Wilson's disease are closely correlated at autopsy in occurrence, appearance and staining characteristics with the lesions of diabetic retinopathy.

Lipaemia Retinalis. Lipaemia retinalis is a rare condition especially since the use of insulin. It occurs in the severe form of diabetes especially in young male adults. When the blood fat rises above 3.5 per cent the vessels take on a lighter salmon color. Blood drawn from the arm has a chocolate color and after standing, a white cream-like layer forms on the surface.

When the blood fat goes above 8 per cent the vessels assume a white appearance as though they contained milk (Fig. VIII). While the condition is looked upon as diagnostic of diabetes it has been reported in 7 non-diabetic patients.

The production of lipaemia retinalis is dependent not upon the total cholesterol being high, but upon the fact that the neutral fat is also high. The condition clears promptly on insulin

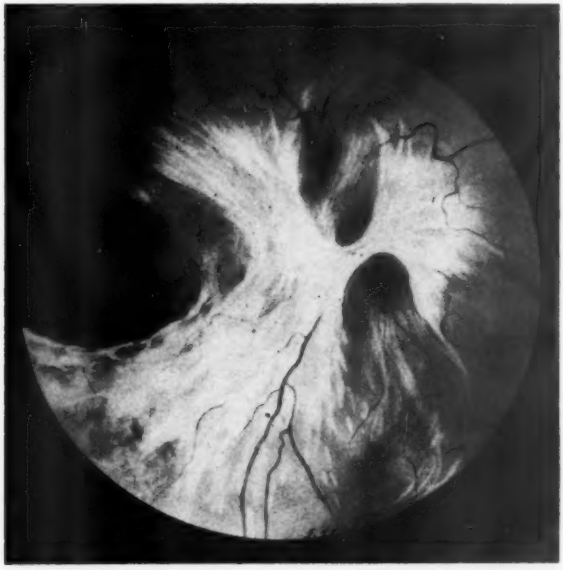


FIG. VII. Diabetic retinopathy, Stage 5, with preretinal hemorrhage and retinitis proliferans (enlarged tracing of fundus photograph).

therapy. Duke-Elder notes that before insulin all 18 cases reported in the literature, with one exception, died in coma.

Optic Atrophy. Walsh says that pallor and atrophy of the optic nerve in diabetes probably depend largely upon widespread arteriosclerotic changes which are part of the disease, and that optic neuritis is rarely causative.

Toxic Amblyopia. Patients suffering from diabetes are particularly liable to toxic amblyopia. Tobacco is especially dangerous to them and explains why the visual acuity at times is lowered out of all proportion to the changes noted in the fundus. The findings are the usual ones of loss of vision and central field changes. Vasodilators are very effective in the treatment of the condition.

Pregnancy and Diabetic Retinopathy. There seems to be a definite relationship between maternal diabetes and congenital defects. Statistics show that the diabetic mother has more than the average liability to abortion, miscarriage, and stillbirth,

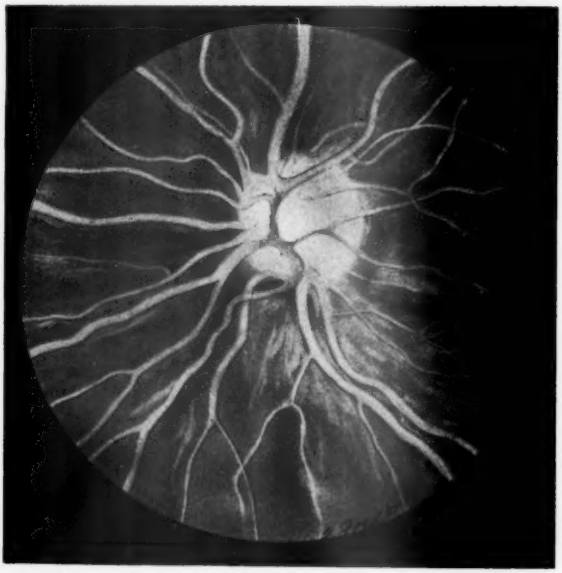


FIG. VIII. Lipemia retinalis in a man aged 29; blood fat, 15 per cent.

especially if the disease is not well controlled. Moreover, the incidence of congenital defects and the mortality of the newborn is twice as high among children of diabetic mothers as it is among children of normal mothers.

Recently Beetham made some interesting observations on the course of pregnancy in the presence of diabetic retinopathy based on a study of 700 pregnant women in Joslin's Clinic. He found that diabetics without retinopathy did well in pregnancy if meticulously controlled; that the presence of any degree of retinopathy reduced the chance of a successful pregnancy to less than 47 per cent; that the prognosis was very poor if a large amount of hemorrhage was present in the fundus of the diabetic; and that no patient with proliferating retinopathy at the onset of pregnancy survived the pregnancy successfully or maintained useful vision. He feels that diabetic wives should have their children as early as possible and certainly before they have had diabetes 20 years.

From Beetham's observations and our limited experience, it would seem that diabetic patients who show extensive retinal homorrhages or proliferating retinopathy should not be permitted to sustain a pregnancy.

Visual Prognosis of the Diabetic. Having considered the ocular complications that occur in diabetes we can now consider their possible effect on the patients vision. The introduction of insulin and a better understanding of the dietary regulations has resulted, according to Joslin, in increasing the life expectancy of the diabetic from 44 to 64 years. Because of the greater longevity more diabetics are developing ocular and other vascular complications. This is particularly true of the juvenile diabetic. In discussing the visual prognosis of the diabetic only two conditions warrant consideration: cataract formation and retinopathy and its complications.

Cataract Formation. Diabetic cataract as a specific condition is rare, occurring in only a few of the younger patients. There is no apparent relation between the blood sugar and the development of the cataract. Linear extraction without iridectomy is the operation of choice in these cases. There are no surgical statistics available in these cases but my personal experience in these cases has been satisfactory, no unusual complications hav-

ing arisen. The visual prognosis is dependent upon the absence or presence of retinopathy.

The surgical prognosis of the senile cataract in the diabetic has been bad in the past, 43 per cent losing their eyes as the result of infection. In addition in about 30 per cent there was serious postoperative hemorrhage. The proper use of insulin and careful control of the patient has changed all this. The presence of small homorrhages is no contraindication to surgery; it should be avoided or delayed, however, in the presence of engorged veins and large retinal hemorrhages. Where opacities prevent even gross visualization of the retina, one must be guided by the light perception and projection. In these cases the possibility of retinopathy must be discussed with the patient and the family.

Where surgery is contemplated certain precautions are indicated. The blood pressure should be controlled as well as possible; all frank foci of infection should be cared for; the diabetes should be controlled as well as possible for several months; and the patient should be hospitalized several days before surgery to control diet and insulin dosage. The blood sugar should not be over 200 mg. per cent. From experience it seems wise to avoid large doses of insulin the day of surgery as it appears to increase the possibility of hemorrhage. If the above precautions are carried out the prognosis is just as good as in the average senile cataract operation. The resultant vision is dependent on whether or not retinopathy is present.

In a series of 600 cataract extractions at the University of California Hospital 71 or 11 per cent were in diabetics. Of this group 53 had no evidence of retinopathy. The visual results were just as good in the diabetics as in the nondiabetics. It is of interest that the percentage of postoperative hemorrhage was lower in the diabetic group than in the hypertensive or arteriorsclerotic group. Thus in the absence of retinopathy the surgical prognosis of cataract surgery in the diabetic is very good.

Diabetic Retinopathy. Diabetic retinopathy with its associated complications, vitreous hemorrhage and retinitis proliferans is by far the commonest cause of visual loss in the diabetic. It is rather generally conceded that the retinopathy is associated with the duration rather than the severity of the disease.

There is no accepted treatment of the retinopathy aside from the treatment of the diabetes. Rutin has been advocated in the treatment of new vessel formation and in cases of hemorrhage. The experience has not been favorable. It would seem to be more successful in vitreous hemorrhages. The most recent account of a new agent to be tried in the treatment of diabetic retinopathy is contained in a preliminary report by Saskin, Walsman, and Pelner. The fact that the incidence of retinopathy is much higher in females than in males suggested that the male harmone might be useful in diabetic retinopathy. The intramuscular administration of testosterone propreinate over a period of from two to six months resulted in the "unequivocal improvement" of more than one-third of the cases, and definite improvement in an additional 40 per cent. Further work is necessary before this treatment can be evaluated.

The visual prognosis in diabetic retinopathy is best considered under two general headings: (1) the prognosis for the juvenile diabetic, and (2) the prognosis for the adult diabetic.

(1) *The juvenile diabetic.* White and Waskow found vascular disease in 93 per cent of 220 juveniles surviving 20 or more years of diabetes; in this group the onset of the disease had occurred before the age of 15 years. Chute found retinitis in all of 24 juvenile diabetics who had survived 20 years. The number of young diabetics who have been kept alive on insulin for 20 years or more is increasing so that more patients with diabetes dating from childhood are now being seen with retinopathy. As already has been stated, in this group the retinopathy is more apt to start with "stage 4," is more severe, and often progresses rapidly to retinitis proliferans. Thus the visual prognosis of the patient who acquires his diabetes in childhood is poor once middle life has been reached.

This increased number of instances of retinopathy in young diabetics who have had the disease for 20 years or more has stimulated new interest in juvenile diabetics, however, and studies indicate that a perfectly controlled diabetic may go through a normal lifespan with a minimum of degenerative changes resulting from the diabetes. This should be a stimulus to both patient and physician to maintain a normal blood chemistry and to treat both minor and major complications early and

adequately, so that the visual prognosis of juvenile diabetics need not be as bad as it has been in the past.

(2) *The adult diabetic.* Among the patients who acquired diabetes in adult life, 73 per cent who had had the disease for more than 20 years developed retinopathy. Dolger studied 200 patients with diabetes of 25 years duration and found all of them with vascular damage and albuminuris or hypertension. There is no doubt that the visual prognosis is bad for those surviving the disease for 25 years, but the number who live so long is relatively small since in the adult the disease occurs most commonly between 40 and 70 years of age. In the entire adult group, according to Martenson and Palm, 60 per cent do not develop retinopathy. In the adult, moreover, there is more tendency for the retinopathy to develop slowly, and many of the patients observed over a number of years show nothing more in the fundus than the early vascular changes, *i.e.*, microaneurysms and small hemorrhages. It is thus apparent that in the adult the older the patient is at the time of onset of the disease, the better his visual prognosis, but if he lives more than 20 years after the onset, the visual prognosis is not good.

The conclusion of Dolger and others that there is vascular damage after 25 years of diabetes in virtually 100 per cent of cases is not shared by Joslin. He feels that the statistically high rate of damage is in part the result of poor treatment and he cites 7 patients who have lived 25 years with diabetes and have shown no signs of degenerative changes in the eyes or the vascular system elsewhere. Sherrill reported a case with onset of diabetes at 14 who had had the disease for 30 years and another with the onset at 16 and of 26 years duration. Neither showed any retinal lesions. In the adult group there were patients who had had diabetes for 21, 22, 22, and 29 years who revealed no retinal pathology. In all of these patients there had been constant and adequate control of the diabetes.

I have seen a patient, a man of 48, who has had diabetes since he was 19 (29 years) who has controlled his disease carefully and constantly. He has normal vision in each eye and his fundi show only a few scattered microaneurysms. Another patient, a woman of 60, has been a diabetic for 31 years (30 on insulin). With the exception of four instances she has been

under perfect control. The vision in each eye is 20/20. The fundi show in the right eye a few scattered aneurysms, a few superficial hemorrhages and some scattered hard exudates. In the left eye there are only a few exudates and a few microaneurysms.

Recently, Keiding and his co-workers reported their findings in a series of 451 patients with onset of diabetes under the age of 30 years and with duration of the disease for 10–36 years. Among 32 patients who had maintained excellent or good control for 20 or more years, none had grade 5 (Wagener) retinopathy and only one had had grade 4 (Wagener) changes. On the other hand in 157 patients, with only fair or poor control, 16 per cent had grade 5 retinopathy and an additional 15 per cent had grade 4. No patient with excellent control had more than minimal retinopathy. It was found that all stages of duration of diabetes the incidence of retinopathy and other complications was significantly less in patients who had maintained excellent or good control. Keiding and his group concluded that only by careful and constant control may the late complication of diabetes be prevented or postponed.

Thus while almost all reports agree that the treatment of diabetes has no effect on the development of the retinopathy, recent scattered reports indicate that the prognosis may not be as black as is now believed, providing the present day methods of treatment are carried out meticulously.

The physician, then, who accepts the diabetic as a patient has a grave responsibility in seeing to it that the patient has all the advantages of modern diabetes therapy. This trust is not to be taken lightly as it may mean, among other things the preservation of the patient's vision.

REFERENCES

Appel, W.: Hochdruck und diabetes. *Klin. Monatsbl. Augenhielk.*, 116: 225–237, 1950.

Ashton, N.: Vascular changes in diabetes with particular reference to the retinal vessels. *Brit. J. Ophth.*, 33: 407, 1949.

Ballantype, A. J., and A. Lowenstein: *Trans. Ophth. Soc. U. Kingdom*, 63: 95, 1943.

Becker, Bernard: Diabetic Retinopathy. *Ann. of Int. Med.*, 37: 273–289, August, 1952.

Beetham, W. P.: Diabetic Retinopathy in Pregnancy. *Trans. Am. Ophth. Soc.*, 48: 205–215, 1950.

Chute, A. L.: Survey of Patients with Juvenile Diabetes Mellitus. *Am. J. Dis. Child.*, 75: 1–10, January, 1948.

Dolger, H.: Clinical Evolution of Vascular Damage in Diabetes Mellitus. *Bull. New York Acad. Med.*, 22: 482–483, 1946.

Duke-Elder, Sir Stewart: Changes in Refraction in Diabetes Mellitus. *Brit. J. Ophth.*, 9: 167–187, 1925.

————: Personal communication.

————: *Textbook of Ophthalmology*. St. Louis, C. V. Mosby, 1941, Vol. 3, p. 2744.

Elschnig, A.: Changes of Refraction in Diabetes. *Med. Klin.*, 19: 17–18. 1923.

Engel, A.: Late Complications of Diabetes Mellitus. *Nord. Med.*, 43: 902–908, June, 1950.

Friedenwald, Jonas: A New Approach to Some Problems of Retinal Vascular Disease. *Trans. Am. Acad. Ophth. and Otol.*, pp. 73–87, 1948–1949.

Joslin, Elliot P.: Furtherance of Treatment of Diabetes Mellitus. *J.A.M.A.*, 139: 1–7, January, 1949.

Keiding, Nils R., Howard F. Root, and Alexander Marble: Importance of Control of Diabetes in Prevention of Vascular Complications. *J.A.M.A.*, 150: 964–969, November, 1952.

Martenson, J.: The Prognosis of Diabetes Mellitus. *Acta Med. Scand.*, 137: 335–354, July, 1950.

Martenson, J., and E. Palm: Ocular Findings in Long Standing Diabetes Mellitus. Report of 163 cases of at least 15 years' duration. *Acta Med. Scand., Suppl.* 246: 154–165, June, 1950.

McCulloch, Clement, and T. J. Pashby: The Significance of Conjunctival Aneurysms in Diabetes. *Brit. J. Ophth.*, 34: 495–505, August, 1950.

O'Brien, C. S., and J. H. Allen: Ocular Changes in Young Diabetic Patients. *J.A.M.A.*, 120: 190–192, September, 1942.

Saskin, E., S. Waldman, and L. Pelner: Diabetic Retinopathy. A New Approach to Therapy with a Steriod Hormone-Testosterone Propionate. *Am. J. Ophth.*, 34: 613–617, April, 1951.

Sherrill, James W.: Diabetic Retinitis: the Relationship Between Retinal Degenerative Changes and the Degree of Diabetic Control. *Bull. Scripps Metabolic Clinic*, 2: 1–17, October, 1951.

Wagener, H. P., and R. M. Wilder: The Retinitis of Diabetes Mellitus. *J.A.M.A.*, 76: 515, 1921.

Wagener, H. P.: Retinopathy in Diabetes Mellitus. *Proc. Amer. Diabetic Assoc.*, 51: 201, 1945.

Walsh, F.: Clinical Neuro-Ophthalmology. Baltimore, The Williams & Wilkins Co., p. 840, 1947.

White, Priscilla and E. Waskow: Clinical Pathology of Diabetes in Young Patients. *South. M.D.*, 41: 561–567, 1948.

FREE AMINO ACIDS IN SEROUS FLUIDS*

J. P. Ellis, W. W. Nowinski, and J. G. Bieri

Ulloa-Gregori, Blocker, Nowinski, and Pomerat (1950) have presented quantitative data showing that the use of ascitic fluid derived from patients with carcinomatosis peritonei as a component of the medium for the cultivation of adult human epidermis *in vitro* is distinctly superior to fluid obtained from patients with non-malignant diseases such as cirrhosis of the liver or congestive heart failure. Results obtained with serum from the human umbilical cord approximate those obtained with ascitic fluid from malignant sources. Cultures treated with autologous or homologous serum from young male adults produced outgrowth only half as extensive as that obtained with the use of the malignant ascitic fluid. In the limited series of quantitative determinations reported by these investigators, no single chemical component, or group of them, could be made responsible for this difference in outgrowth. The advantage of the use of serous fluids also was substantiated in the further work of Pomerat, Nowinski, and Rose (1950). An accumulation of data in the form of routinely performed quantitative analyses is being carried out in order to increase our knowledge of the chemical composition of various types of body fluids used in tissue culture experiments.

Although our data relative to the distribution of amino acids in ascitic fluid is limited, it was considered worth-while to make the information available to workers in the many laboratories where serous fluids are being employed as a constituent of tissue culture media. Moreover, the amino acids required for the development of synthetic media is a subject of current importance. The importance of these results also seemed to be of particular interest due to work of A. Fischer (1941, *see also* Fischer, 1947) who claimed that explants *in vitro* do not grow in a homologous medium composed of intact proteins only; that free amino acids have to be added to the medium and that among

* From the Tissue Culture Laboratory, Tissue Metabolism Research Laboratory and Department of Biochemistry and Nutrition, The University of Texas Medical Branch, Galveston. Aided by a grant from the American Cancer Society CP-12E administered by Dr. C. M. Pomerat. Received for publication March 2, 1953.

them, the presence of cystine is a *conditio sine qua non* for normal growth (Fischer, 1948).

Free amino acids were estimated by partition chromatography. Whatman paper No. 1 or No. 4 was used in sheets for the ascending chromatograms. The solvents for the two dimensional analysis were phenol (redistilled) and 2, 4-lutidine, both saturated with water (Fig. 1).

FIG. I. A typical two dimensional chromatogram of ascitic fluid.

The preparation of samples was carried out in the majority of cases, according to the method described by Awapara (1948). The serous fluids were treated with alcohol in order to precipitate the proteins, centrifuged, and the alcohol removed with

chloroform. Some samples also were obtained by dialysis against distilled water. In both cases, the quantity of amino acids present turned out to be so small that a ten to fifteen fold concentration of the fraction was necessary. This was carried out either by evaporation under vacuum over P_2O_5 at room temperature, or by evaporation at 60° C. in a partial vacuum.

Table 1 summarizes the types of serous fluids used, together with the diagnoses. Our estimations consist of 7 malignant and

TABLE 1

Serous Fluids Used in the Study of Amino Acid Components

Patient	Fluid	Diagnosis
TU.....	Ascitic.....	Cirrhosis of liver
CL.....	Ascitic.....	Adenocarcinoma of ovary
MI.....	Ascitic.....	Carcinoma of transverse colon
ST.....	Ascitic.....	Adenocarcinoma
FU.....	Pleural....	Malignant cells in pleural fluid; squamous cell carcinoma from vaginal vault
MO.....	Pleural....	Metastatic adenocarcinoma near left axilla
RO.....	Pleural....	Hepatoma mixed neoplasm of parotid gland
WO.....	Pleural....	Carcinoma of pelvis (?) empyema
McCL...	Ascitic.....	Congenital heart failure
WI.....	Ascitic.....	Cirrhosis of the liver

3 non-malignant serous fluids. Two of the ascitic fluids were previously tested for their outgrowth promoting activity *in vitro* (see Ulloa-Gregori, Blocker, Nowinski, and Pomerat, 1950: Fig. IV, patient ST and Fig. V, patient MI).

Results of the estimations are summarized in Table 2. The following amino acids were present in all the fluids:

Aspartic acid	Methionine
Glutamic acid	Leucine
Serine	Glycine (except in the case of WA)
Proline	Tyrosine
Valine	

The pleural fluid of one patient (WA) proved to be particularly poor in amino acids because it contained only seven of the compounds listed. In some ascitic fluids, alanine (TU, CL, FU, RO, McCL, and WI) was present.

The presence of glutamine, arginine, and histidine was questionable in 4 cases, negative in 3 cases, and definitely present in 2 cases. The comparison between the malignant and non-malignant exudates and of ascitic versus pleural fluids do not reveal any striking differences. For instance, the composition of ascitic fluid of the patient TU (cirrhosis of the liver) is almost identical with that of patient CL (adenocarcinoma of the ovary) and of the others.

TABLE 2

Amino Acid Content of Serous Fluids

	TU	CL	MI	ST	FU	MO	RO	WA
Aspartic Acid.	+	+	+	+	+	+	+	+
Glutamic Acid	+	+	+	+	+	+	+	+
Serine	+	+	+	+	+	+	+	+
Glycine	+	+	+	+	+	+	+	—
Alanine	+	+	—	—	+	—	+	—
Glutamine ...	?	—	—	—	?	—	?	—
Arginine	—	—	?	—	?	—	?	—
Histidine	?	—	?	—	?	—	?	—
Proline	+	+	+	+	+	+	+	+
Methionine ...	+	+	+	+	+	+	+	+
Leucine	+	+	+	+	+	+	+	+
Tyrosine	+	+	traces	traces	+	traces	+	traces

The amino acid fractions of two malignant fluids (MI and ST) were evaluated in tissue culture medium consisting of embryonic extract, Gey's balanced salt solution and the amino acid fraction in place of the plasma used for the controls. Skin explants were employed for the assay of their activity, but no effect on outgrowth was observed. According to Fischer (1941), the following amino acids are essential for the normal outgrowth of fibroblasts:

Lysine	*Glutamic Acid
*Arginine	*Aspartic acid
Tryptophan	*Proline
*Methionine	Cystine
*Histidine	

The amino acids identified by us in serous fluids are marked with an asterisk (*). Whether or not the serous fluids contained

tryptophane and cystine is very difficult to establish because both of these amino acids do not show up easily in partition chromatography. The fact, however, that the fractions investigated do not possess outgrowth promoting activity *in vitro* leads us to assume that the active agent is probably of a different nature and not connected with the free amino acid contents. In the tissue culture tests, the serous fluids were combined with embryonic extract and therefore the possibility exists that this combination of nutrients forms a particularly favorable substrate for the stimulation of the outgrowth.

Summary

Amino acids of a small number of specimens of serous fluids were analyzed by paper chromatography. The presence of the following amino acids was demonstrated: aspartic acid, glutamic acid, serine, glycine, proline, methionine, and leucine. In some cases the following amino acids were questionable or negative: alanine, glutamine, arginine, and histidine. Tyrosine was definitely present in half of the cases and only traces were found in the other half. No significant difference was found between those of the malignant and the non-malignant series. Tests in tissue cultures showed that the amino acid fraction alone is not accountable for the outgrowth promoting activity.

REFERENCES

Awapara, J.: Application of paper chromatography to the estimation of the free amino acids in tissues. *Arch. Biochem.*, 19: 172–173, 1948.

Fischer, A.: Die Bedeutung der Aminosauren fur die Gewebezellen *in vitro*. *Acta Physiol. Scand.*, 2: 143–187, 1941.

————: The participation of blood proteins in the metabolism of tissue cells. *Biol. Rev.*, 22: 178–194, 1947.

————: Amino-acid metabolism of tissue cells *in vitro*. *Biochem. J.*, 43: 491–497, 1948.

Pomerat, C. M., W. W. Nowinski, and G. G. Rose: Tissue cultures of cells from body fluids. *Texas Rep. Biol. and Med.*, 8: 521–532, 1950.

Ulloa-Gregori, O., T. G. Blocker, Jr., W. W. Nowinski, and C. M. Pomerat: The effect of ascitic fluid and of blood serum on the outgrowth of epidermal cells from the human skin in tissue culture. *Texas Rep. Biol. and Med.*, 8: 400–409, 1950.

THE EVOLUTION OF ESOPHAGOPLASTY

WESLEY FRY*

Many ingenious solutions have been offered for the problem of constructing an artificial esophagus following the resection or exclusion of this organ for obstructing disease. The historical facts relating to this question bespeak an evolution of ideas which have culminated in those methods presently in use, and those which have been proposed, for esophageal replacement.

The simplest method of relieving an obstruction of the lower intestinal tract by surgical means, and therefore the first to be employed, was the creation of an opening the gut, proximal to the obstruction, for the purpose of permitting egress to the intestinal wastes. Littre (Dinnick, 1934), in 1710, was the first to propose colostomy for treatment in a case of imperforate anus. It remained for Pillore (Dinnick, 1934) in 1776, however, to perform this operation. Similarly, the simplest procedure for relief of an obstruction high in the alimentary tract was the making of an ostium in the tract distal to the obstruction, for the purpose of feeding the patient. It was not until 1875 that Jones (1875) performed the first successful gastrostomy in man. Prior to this, 32 attempts at gastrostomy by other surgeons had ended fatally.

While abdominal surgery was advancing, there was a great impediment to progress in surgery of the thorax, namely, the imperfect knowledge of respiratory mechanics. No method was available to maintain the respiratory equilibrium when the chest was widely opened. The physiologists were performing operations on the chests of animals by means of artificial respiration with alternating variation in air pressure delivered through a tracheotomy. Some operations were performed on the pleura (empyema drainage), pericardium (drainage of purulent effusion), heart (suture of wounds), and lungs (removal of cysts). Intrapleural operations were successful only when the pleural

* Commander, M.C., U.S.N., Oakland, California. The opinions expressed are those of the author and are not to be construed as official or reflecting the views of the Navy or the Naval Service at large. From the Department of Surgery, University of Michigan. Received for publication January 15, 1953.

space had been sealed by loculation or symphysis, so that a total pneumothorax did not occur. A wide opening of the pleura was often followed by catastrophe. The esophagus could not be approached transpleurally without lethal consequence.

In 1886, according to Frangeheim (1913), both Mikulicz and von Hacker made partial esophagoplasties in the cervical region to bridge the gap after local resection of the esophagus and larynx. The von Hacker (1914) method consisted of making a tube from quadrilateral skin flaps from the anterior aspect of the neck. The tracheotomy wound was left open just anterior to the lower end of the artificial gullet.

In the same year, von Mikulicz (quoted by Fischer (1923)) tried an anterior approach to the esophagus but was not successful. In 1886, Nasiloff (Nasiloff, 1888) described an extrapleural approach to the esophagus which overcame the hazard of pneumothorax. The operation, although dangerous and difficult, was an important advance in thoracic surgical technique. He advocated an incision parallel to the spinal column on the left posterior thorax, with the patient prone. At each end of this incision a transverse incision was made, thus forming a flap. The ribs were divided posteriorly and pieces resected. The pleura was pushed away to expose the posterior mediastinum and the esophagus.

According to E. Bircher (1907), H. Bircher in 1894 employed an original esophagoplastic procedure using a skin tube to connect the cervical esophagus with a gastrostomy. The skin tube was formed in situ on the anterior chest wall, and covered by lateral skin flaps (Fig. I). This esophagodermatogastrostomy is the simplest concept in esophagoplasty and hence, logically, was the first to be devised. Except for the simplicity of design, however, this procedure is one of the most difficult of the plastics used for reconstruction of the esophagus. Bircher's operation was performed in 3 stages. His 2 patients died.

Abbe (1894) published his method of cutting esophageal strictures with a string. The swallowed string was pulled out through a gastrostomy, and by traction alternately on this end and the end protruding from the mouth, the fibrous stricture was cut enough to permit dilatation with a bougie.

Much experimental work was done on the esophagus in dogs during the closing years of the last century. One of the principal problems at that time was end-to-end anastomosis after resection of a portion of the esophagus, or gastroesophagostomy after resection. Partial success was achieved in work on this problem. Dobromysslow (1901) succeeded in anastomosing the esophagus and stomach by suture. Sauerbruch (1905) was more successful with a button technique, similar to that used by Murphy for intestinal anastomosis.

Biondi (1895) presented his "experimental method" of pulling the fundus of the stomach up through an incision in the diaphragm, making an anastomosis between the fundus and the proximal stump of the esophagus after a segment of the latter organ had been resected (Fig. II). This was the first intrathoracic substitution for the esophagus and was the antecedent of modern gastroesophagostomy. No experimental data were offered.

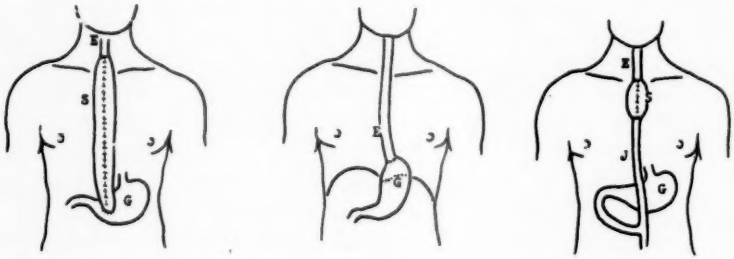


Fig. I Fig. II Fig. III

FIG. I. H. Bircher (1894). E, esophagus; S, dermal tube; G, stomach.
FIG. II. Biondi (1895). E, esophagus; G, stomach.
FIG. III. Wullstein (1904). E, esophagus; S, dermal tube; J, jejunum; G, stomach.

Levy (1898) devised a method for resection of the esophagus without entering the thorax. The technique consisted of dividing the esophagus in the neck and inverting its lower segment by means of a string passed down through its lumen and out a gastrostomy. After the esophagus had been pulled down, it was cut off. Levy developed this method in dogs, but after cadaver experimentation, he decided it was dangerous for human use.

During the same year, Matas (Keyes, 1945) recommended that anesthesia for thoracic surgery be given by intrapharyngeal insufflation, after the principle of Meltzer and Auer. The idea of Matas was a signal contribution to the solution of the basic problem of opening the thorax safely, but it was several years before his advice was accepted and put into practice by other surgeons.

Kelling (1904) dissected the esophagus transthoracically, closed the chest wall and brought the esophagus out in the neck after it had been divided in its lower part. This intrathoracic work was done under artificial respiration.

Gosset (1903) repeated the work of Biondi on cadavers and dogs. He recommended lateral anastomosis of the stomach and esophagus. He also mentioned excluding the cardia by anastomosis of the stomach and esophagus around a lesion of the cardia. He mentioned use of the transthoracic and transdiaphragmatic route for total gastrectomy and anastomosis of the esophagus and jejunum.

Wullstein (1904) reported a procedure for making an artificial esophagus by mobilizing a loop of jejunum and bringing it out anterior to the sternum, subcutaneously, to connect with a dermal tube, which was in turn anastomosed to the cervical esophagus (Fig. III). This was the first instance of antethoracal esophagoplastic operation involving intestine as a part of the new esophagus.

Beck and Carrell (1905) were the first to offer a plastic operation on the stomach for construction of an artificial canal for feeding when the esophagus is obstructed. They described a method of tubular gastrostomy, the tube being fashioned from a flap of the greater curvature of the stomach and lined with mucosa. As recently as 1941, the Beck type of gastrostomy was considered by Sweet (1941) to be the most satisfactory method he had tried in carcinoma of the esophagus. Beck and Carrell also showed how this type of gastrostomy could be combined with a dermal tube to make an antethoracic esophagus (Fig. IV a and b).

Murphy (1905) developed a device for positive pressure anesthesia. The patient's head was placed in a box and a positive pressure maintained in the box through which ether

was also administered. This permitted safe opening of the thorax. Slight attention was paid to Murphy's method; perhaps because it was published in the United States where little thoracic surgery was being attempted at the time.

The following year, Sauerbruch (1906) published an account of his differential pressure machine for artificial respiration. This apparatus consisted of a negative pressure chamber in which the surgeon and assistant worked. The patient's body was in this chamber but his head was outside at atmospheric pressure. The idea was practical but the apparatus was cumbersome and the working room in the chamber was inadequate. Still this invention of Sauerbruch gave a great impetus to thoracic surgery and within the next year numerous treatises on esophageal work were published. At last the surgeon had a means at his disposal whereby the chest could be opened without immediate danger of his patient's death.

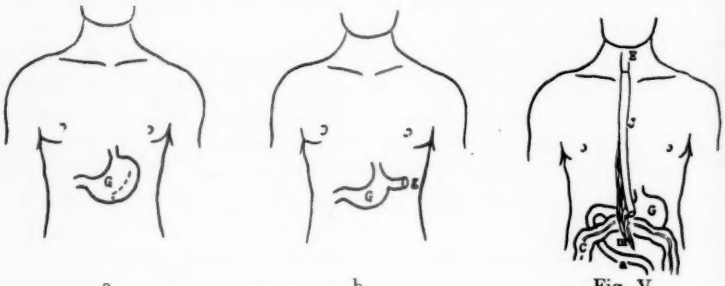


Fig. IV Fig. V

FIG. IV. Beck and Carrell (1905). *a* G, stomach—dotted line indicates incision. *b* G, stomach; g, gastrostomy stoma.

FIG. V. Roux (1907). E, esophagus; J, transplanted jejunum; G, stomach; m, mesentery of loop J; a, anastomosis of jejunum.

Green (1906) also published his technique of intratracheal, positive pressure anesthesia, using a cannula with an inflatable balloon to insure a tight fit with the tracheal wall. He also reported investigations pertaining to intrathoracic operations in 14 dogs, using his positive pressure method. This is essentially the manner in which the patient's breathing and anesthesia are maintained in present day intrathoracic work. This procedure did not gain immediate popularity, as Sauerbruch's did, prob-

ably because the name of Sauerbruch, even at that time, carried great weight among the European surgeons who were doing most of the pioneer work in this field.

Tavel (1906) recorded his technique for gastrostomy, using a short segment resected from the jejunum but with its vascular pedicle intact, as a connecting tube between the gastric lumen and the exterior. This procedure was the antecedent of the Lexer esophagoplasty.

Roux (1907), in the same year, proposed the use of an isolated loop of jejunum, with vascular supply preserved, as a total replacement of the esophagus. The loop of jejunum was used as a subcutaneous antethoracic esophagus, being anastomosed to the cervical esophagus at one end and to the stomach at the other. The ends of the jejunum from which the loop was removed were joined by an anastomosis (Fig. V). The following year, Herzen (1908) suggested a modification of the Roux method of jejunal esophagoplasty by dividing the jejunum only once. The distal segment was passed subcutaneously to the neck and anastomosed to the cervical esophagus. A side-to-side gastrojejunostomy was made and the proximal stump of the jejunum was joined to the distal jejunum (end-to-side) below the gastrojejunostomy. The loop of jejunum forming the new esophagus was passed through the transverse mesocolon and gastrocolic ligament (Fig. VI).

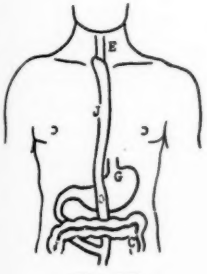


Fig. VI

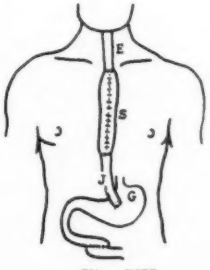


Fig. VII

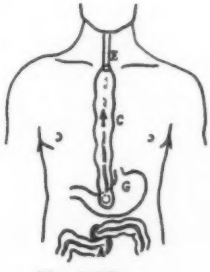


Fig. VIII

FIG. VI. Herzen (1908). E, esophagus; J, jejunum; G, stomach; C, colon.
FIG. VII. Lexer (1911). E, esophagus; S, dermal tube; J, jejunal transplant; G, stomach.
FIG. VIII. Vulliet (1911). E, esophagus; C, transplanted transverse colon; G, stomach; A, colic anastomosis: Arrow indicates direction of peristalsis.

During this year, Voelcker (1908) published an account of the first successful resection of the abdominal esophagus. The following year, Kummel (1922) reported another successful resection of the abdominal esophagus.

Meyer (1909) published his investigations on esophagogastrostomy in dogs after resection of the esophagus. His operation was much like the modern esophagogastrostomy.

Lexer (1911) also proposed a technique for esophagoplasty. He used a short loop of jejunum, as in the case of Tavel's gastrostomy, and indirectly joined the outer end of this loop to the esophagus in the neck by means of a dermal tube (Fig. VII).

Two surgeons in the year 1911 reported the use of the transverse colon as an antethoracic replacement of the esophagus. Vulliet (1911) used the transverse colon antiperistaltically to connect the cervical esophagus with the stomach (Fig. VIII). Kelling (1911) employed the transverse colon for the same purpose, but in an isoperistaltic manner. He also used a dermal tube between the esophagus and colon (Fig. IX). The transplanted colon was supplied with blood by the middle colic vessels.

Jianu's (1912) esophagoplasty and gastrostomy were described in 1912. The procedure was essentially the same as that of Beck and Carrell in 1905. This is mentioned only to invite attention to the fact that the method frequently referred to as Jianu's esophagoplasty is so called erroneously.

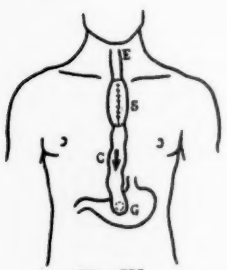


Fig. IX

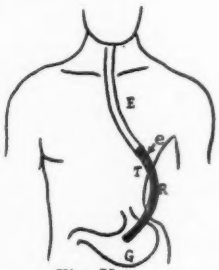


Fig. X

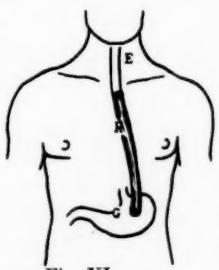


Fig. XI

FIG. IX. Kelling (1911). E, esophagus; S, dermal tube; C, transverse colon; G, stomach: Arrow indicates direction of peristalsis.

FIG. X. Zaaijer (1913). E, esophagus; e, posterior esophagostomy; T, lower thoracoplasty; R, rubber tube; G, stomach.

FIG. XI. Torek (1913). E, esophagus; R, rubber tube; G, stomach.

Zaaijer (1913) described his method of esophagoplasty and announced the third case of successful resection of the cardiac esophagus. The esophagoplasty consisted of a preliminary gastrostomy, extensive thoracoplasty of the lower chest, followed by resection of the lower esophagus and bringing the proximal stump to the skin in the posterior axillary line. This stump he connected with the gastrostomy (Fig. X) by means of a tubular rubber prosthesis.

Torek (1913) published his now famous case of the first successful resection of the thoracic esophagus for carcinoma. Eleven years later, he published (1925) a follow-up of this case, at which time the patient was alive and well. The patient died 2 years after this report, without recurrence. The technique employed by Torek was transthoracic resection of the esophagus with closure of the lower end and exteriorization of the proximal stump in the neck. He used a rubber tube as an artificial esophagus to connect the proximal stump with a gastrostomy (Fig. XI). The work of Torek revived hope for the cure of cancer of the thoracic esophagus. As in the case of the first successful gastrostomy, this success encouraged many other surgeons. As late as 1940, Carter (1940), said, "For the complete removal of a majority of the carcinomas of the thoracic esophagus, the Torek procedure is the most satisfactory. Esophagogastrostomy finds its greatest field of usefulness in the treatment of carcinoma of the cardiac end of the stomach or of carcinoma of the terminal esophagus." However, 10 years later, we have almost abandoned the Torek operation, and esophagogastrostomy as advocated by Garlock (1944), Adams (1938), and Sweet (1945), is used now almost to the exclusion of other methods. The incision used by Torek traversed the seventh left intercostal space, and its posterior end extended upward, severing the posterior parts of ribs 7, 6, 5 and 4.

Ach (1913) developed a method of removing the esophagus by blunt dissection from abdominal and cervical approaches. After this dissection, the lower end of the esophagus was severed, and an instrument he had devised was passed down through the pharynx and esophagus; the lower end of the proximal segment of esophagus was then fixed to the instrument and the instrument was withdrawn through the mouth. The esophagus be-

came invaginated and was delivered from the mouth. When the lower end of the instrument emerged, the instrument was released and the esophagus was extracted through the neck wound. Ach's method differed from that of Levy only in the direction of stripping out the esophagus.

The obvious dangers in the Levy-Ach method of extirpation of the esophagus are from infection, from hemorrhage and pneumothorax. These conditions result from tearing the esophagus where weakened or fixed by a lesion, from tearing the several arteries to the esophagus which arise directly from the aorta, and from tearing the pleura, respectively. Such hemorrhages were reported by Rhen (1914), in dogs. These dangers he overcame by stripping out only the mucosa. In man, he was able to remove the esophagus by inversion downward, as in Levy's method. There is also a more remote danger of tearing the vagi and the recurrent nerves.

Frangenheim (1913) and Blauel (1916) published reviews of the techniques that had been used for esophagoplasty to date. Blauel offered a modification of Wullstein's method that differed only in that there was a gastrojejunostomy in the loop of jejunum used to connect with a dermal tube. In this respect, it resembled the Herzen modification of the Roux esophagoplasty (Fig. XII).

In 1913, Fink's (1913) method (Fig. XIII) of esophagoplasty was published. He mobilized the stomach at the pyloric end and divided the first part of the duodenum. The distal stump of the severed duodenum was closed. The stomach was then rotated into an antiperistaltic position and the proximal duodenal stump anastomosed to a subcutaneous dermal tube which, in turn, was anastomosed to the oral end of the divided esophagus. A gastroenterostomy was a necessary complement to this procedure in order to restore continuity to the alimentary canal.

Several years later, Razzaboni (1919) employed free intestinal grafts in cats and dogs as a substitute for a resected portion of the esophagus. Some of these grafts were autogenous, others were homogenous. The grafts were successful at first but later shriveled and contracted to a fraction of their original lumenal dimensions.

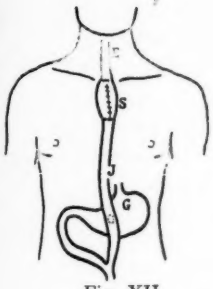


Fig. XII

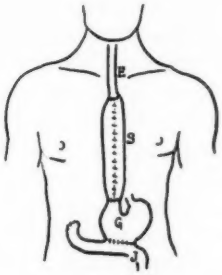


Fig. XIII

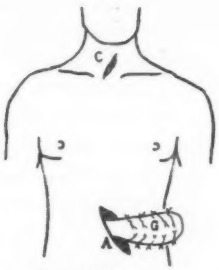


Fig. XIV—a

FIG. XII. Blauel (1916). E, esophagus; J, jejunum; G, stomach.
FIG. XIII. Fink (1919). E, esophagus; S, dermal tube; G, stomach; J, jejunum.
FIG. XIV. Kirschner (1920). *a* C, cervical incision; A, abdominal incision; G, mobilized stomach. *b* E, oral stump of esophagus; E′, aboral end of esophagus; J, jejunum.

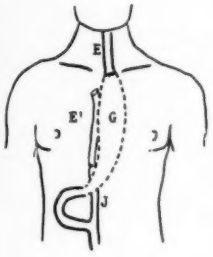


Fig. XIV—b

Fig. XV

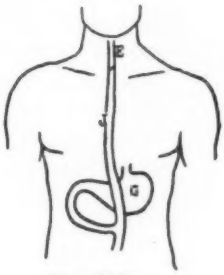


Fig. XVI

FIG. XV. Abdominothoracic incision of Kirschner (1920). The dotted line indicates the incision.
FIG. XVI. Yudin (1944). E, esophagus; J, jejunum; G, stomach.

Kirschner (1920), performed subcutaneous, total esophagoplasty by using the stomach to replace the esophagus. He mobilized the stomach from the left, by severing and ligating the vasa brevia, the left gastroepiploic and the left gastric artery. He then brought the stomach up, subcutaneously, to meet the oral stump of the esophagus (Fig. XIV).

He also offered a contribution to esophagogastric anastomosis, in that he sealed this anastomosis against leaks by wrapping the redundant fundus of the stomach around the suture line.

Kirschner proposed an abdominothoracic incision which, with modifications, is still considered one of the best for the exposure of the thorax and upper abdomen (Fig. XV).

Kummel (1922) demonstrated removal of the esophagus without opening the chest. He mobilized the organ by finger dissection through abdominal and cervical incisions. The stomach was next pulled up into the chest by traction on the esophagus. Resection of the esophagus was then accomplished and esophagogastrostomy was performed in the neck. Thus, a total intrathoracic esophagoplasty was made from the stomach for the first time.

Neuhof (1922) used granulation tubes experimentally for replacement of part of the esophagus. His 2-stage procedure was successful and the granulation tract he established became lined with esophageal epithelium.

Engelbrecht (1922) wrote of a case requiring 20 operative procedures to produce a suitable replacement for the esophagus. He thought mistakes in this case confirmed the advantage of forming a skin tube extending from the larynx to the gastric fistula. When the dermal tube healed, he connected it to the esophageal stump in the neck. Temporarily, he used a rubber tube to connect the lower end of the dermal tube to the stomach. After healing of the skin in the vicinity of the fistula, a flap was raised to close the gap between this opening and the dermal tube.

Nicolaysen (1924) prepared a review of the techniques of esophagoplasty up to that time. In the same year, Roith (1924) reported a case of esophagoplasty, in which the patient lived for 3 years after an antethoracal colon transplant had been done. The patient died of intercurrent disease.

Sauerbruch, in 1925, inverted a segment of esophagus containing tumor into the stomach, after devitalizing it. The devitalized mass was digested and sloughed into the stomach. An extrapleural approach was used for this operation.

Also Eggers (1925) reported the successful resection of the thoracic esophagus for carcinoma, using the Torek method.

Reisenkampf (1925) constructed an esophagodermatogastrostomy by a method combining the 3 stages used by Bircher into a single procedure.

Galpern (1925) used omentum for sealing the anastomosis of a dermatogastric tube. This method of sealing the suture line and promoting healing was very successful in dogs.

The same year, Allen (1922, 1925) was the first to reconstruct the entire circumference of the esophagus by using facia lata. In carcinoma of the esophagus, he wrapped a sheet of fascia around that portion of the esophagus containing the tumor. Loose ligatures were placed around the esophagus above and below the tumor, and the sheet of fascia sutured to form a tube. At a second stage, the ligatures were tightened to devitalize the esophageal segment containing the tumor. The fascia lata formed a suitable substitute for the gullet. Stenosis developed later at the site of the fascia plastic, but as Allen indicated, the exchange of a stenosis for a carcinoma is worth while.

Rovsing (1925) in 1926 published his method of esophagoplasty in several journals. His method is essentially that of Bircher. Singularly enough, no reference to Bircher is made by Rovsing, although many other writers had made reference to the Bircher technique long before Rovsing's papers appeared.

Rudolf (1929) presented his method of total extrathoracic esophagoplasty by utilization of the right colon.

Turner (1934) excised the thoracic esophagus without entering the chest. His operation was similar to that of Levy and Kummel in regard to the extirpation of the esophagus. During the dissection, the pleura was entered by accident, a mishap which is to be expected in a blind maneuver involving the delicate pleura and a possibly adherent esophagus. Turner replaced the esophagus by an antethoracic tube made from skin and jejunum to which he refers as the method of Rovsing.

J. Jiano (1938) developed a new technique for forming a dermal-tube, antethoracic esophagus. He raised one skin flap from the abdomen and another from the left scapular region. These flaps he formed into dermal tubes which he passed subcutaneously in the anterior chest wall. An incision was made in the skin at the place where the 2 tubes met, and through this incision, the lumens of the tubes were anastomosed. Then they were connected with the cervical esophagus and the gastrostomy. Part of his technique was the exteriorizing of the gastric fundus in the shape of a cone, which was passed through an incision

in the anterior abdominal wall, after a chondral resection. This was his method of forming a gastric ostium to connect with the cutaneous esophagus.

Garlock (1940) published his method of esophagogastric anastomosis in which the proximal esophageal stump is intussuscepted into the mobilized stomach.

Rapin (1941) proposed the construction of an antethoracic esophagus by forming a dermal tube from split skin grafts which he placed in a tunnel beneath the skin of the sternal region. This was a distinctly new modification of the dermal tube technique.

Ochoner and DeBakey (1941) published a method for constructing a long tube from the stomach for use in esophagel replacement.

Many articles were published in 1942 on the subject of esophagogastrostomy. The sudden interest in this procedure was perhaps due largely to the success and publications of Garlock (1938, 1940) in this field.

Kay (1943) published the results of his research on dogs. He employed a tube formed from the anterior wall of the stomach after the manner of the tube constructed by Beck. However, he formed an intrathoracic esophagus from it instead of the less satisfactory subcutaneous one made by Beck, Jianu *et al.*

Miscall and Clark (1943) used a Murphy button in esophagogastric anastomosis, and advised reinforcing the union with interrupted silk sutures. This is similar to Sauerbruch's technique of 40 years previously.

Yudin (1944) published a report on 80 cases of esophagoplasty. He perhaps has the greatest personal experience of any surgeon in this field. Yudin has used many types of esophagoplasty but prefers a jejunal esophagoplasty as used by Herzen, but without gastroenterostomy (Fig. XVI).

Sweet (1945) wrote of the inadequacy of the Torek procedure. No abdominal lymph nodes are removed in the Torek operation. Sweet's procedure requires opening the diaphragm to accomplish mobilization of the stomach and a complete removal of regional nodes.

Allison (1946) recommended esophagojejunostomy for the exclusion of carcinoma of the gastric cardia.

Longmire and Ravitch (1946) made an esophagoplasty which was a departure from anything previously attempted. In dog experiments, and later in human subjects, they buried a jejunal loop with a vascular pedicle subcutaneously. Later a dermal tube was constructed around the segment of intestine. At another stage, the tube was raised and the mesenteric blood supply was divided. When circulation had become adequate, the extracorporeal tube with its mucosal lining was switched and anastomosed to the esophagus and stomach. The skin tube was then opened and implanted along the anterior chest wall.

Rienhoff (1946) described his operation of intrathoracic esophagojejunostomy for replacement of a resected portion of the esophagus. He reported 3 successful cases in which this procedure was employed.

H. Brodie Stephens (personal communication) has used this technique unsuccessfully twice with the transplanted jejunal loop in the antiperistaltic position. However, he has been more successful when the jejunum is used isoperistaltically.

Hanrahan (1947) published a method using migrated tubular grafts, both to cover and line a dermal-tube esophagus. This method was originated by J. S. Davis (1942) who constructed an antethoracic esophagus from a short segment of jejunum and a dermal tube (made in situ from the skin of the thorax). Rather than mobilize flaps of the chest to cover the new esophagus, he accomplished this by means of a migrated tubular skin flap.

Swenson (1947) reported the use of anterior esophagoplasty in esophageal atresia and tracheoesophageal fistula. He advocated gastrostomy and marsupialization of the esophagus in the neck. After animal experimentation, he employed a subcutaneous jejunal loop to form the prethoracic esophagus. He observed a difference in the mesenteric vascular pattern in animals and man. In man, the vascular arrangement is much more constant and reliable than in dogs. Swenson's first 12 animals died because of inadequate circulation in the jejunal loop which was placed subcutaneously.

Longmire (1947) anastomosed the internal mammary vessels to the mesenteric vessels of a subcutaneous jejunal loop. He stated that certain patterns of the vessels may make a sufficient length of jejunum impossible for the purpose of constructing

a total esophageal replacement. The technique of anastomosis of the internal mammary vessels with the mesenteric vessels is brilliant but difficult to perform. But, as Longmire pointed out, it is questionable whether the anastomosis actually functions. No use of angiography was mentioned in regard to determining the patency of the vascular union.

A new operation was devised by Mes (1948) for extrathoracic esophagoplasty. Due to its distensibility, a very long tube can be fashioned from the greater curvature and fundus of the stomach. The gastroepiploic arteries have an undulating course which allows for great expansion of the greater curvature. The right and left gastric arteries are short and straight; hence, they inhibit stretching of the lesser curvature of the stomach. When the lesser curvature is excised or separated by longitudinal incision from the greater curvature, a tube can be made from the latter portion which has a good blood supply and will not slough. The tube makes a very satisfactory antethoracic esophagus (Gig. XVII). This technique is similar to that of Ochsner and DeBakey (1941).

Kresmen (1948) described an improvement of the Kirschner abdominothoracic incision. Kresmen's incision extended along an intercostal space and cintinued across the cartilage into the abdomen without the deviation of the Kirschner incision. In the same year Reynolds and Young (1948) advocated the use of a Roux-Y jejunal plasty for circumventing carcinomatous growths of the stomach and lower esophagus. The idea was the same as that of Allison (1946) but the technique differed.

Cristophe (1951) reported the successful use of the transverse colon for constructing a subcutaneous isoperistaltic esophagus in a case of esophageal fistula.

Orsoni and Lemaire (1951) also wrote of their studies in the use of the transverse and descending colon in cadavers and concluded that colon is the best material for esophagoplasty. They proposed its use in prethoracic and intrathoracic situations. In the latter case, they mentioned substernal, anterior mediastinal, and right and left posterior intrapleural positions for the artificial esophagus. They then proposed to make clinical use of this method. Instructions were given for the procedures they had worked out.

Rudler (1951) also made inquiries into the data from the experiences of 6 groups of French surgeons on procedures and results in esophagoplasty, using the colon as the substitute for the esophagus. This series consisted of 28 cases of human prethoracic esophagoplasty, including 9 made from the right colon and 19 from the transverse colon, the colon in all cases being brought up to the neck where it was anastomosed to the cervical esophagus. Fistulas developed at the esophagocolic junction in 6 of the 8 surviving patients in whom the right colon was used. Ten patients of the surviving 12 having transverse colon transplants developed fistulas. Two of the fistulas closed spontaneously as compared with 14 which did not. It appears, therefore, that cervical fistula is a very serious complication in antethoracic colonic esophagoplasty, judging from the high percentage of fistulas encountered in Rudler's series. The method of anastomosis between the esophagus and colon or terminal ileum appeared to be of no consequence. The 21 per cent mortality was considered by Rudler not to be excessive since the surgeons were dealing with a fatal disease and in most cases palliation only was sought.

Fig. XVII

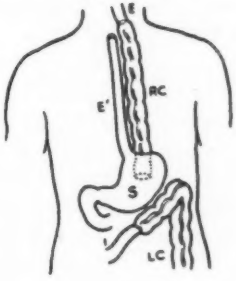


Fig. XVIII

FIG. XVII. Redrawn from Mes, showing his incision of the stomach for the construction of a long gastric tube for esophagoplasty (dotted line). Shaded area not used in the plastic procedure.

FIG. XVIII. E, oral esophagus; E', aboral esophagus; RC, right colon; S. stomach; I, ileum; LC, left colon. (By permission of *Surgery, Gynecology and Obstetrics.*)

Recently the author (Fry 1953) has proposed a method for intrathoracic isoperistaltic colonic esophagoplasty, based upon

experimental work, using the ascending colon to replace the resected esophagus. This method is of doubtful value in the treatment of carcinoma but should be effective in certain other benign obstructing lesions of the esophagus (Fig. XVIII).

Robertson (1952) *et al.*, reported a case 4 years after operation in which the left colon was used to replace the lower esophagus and stomach in man with satisfactory results.

Hushang (1952) has used aortic grafts experimentally to bridge defects in the esophagus. These defects were from 2 to 7 cm. in length. Healing and epithelization of the lumen occurred and adequate patency was demonstrated by X-ray and barium swallow. Both fresh and preserved homologous grafts were used.

Summary and Conclusions

It is believed that the foregoing discussion may clarify the history of esophagoplasty and serve as an outline to the voluminous literature on the subject.

In several instances the publication of procedures duplicating those already documented in the literature were found by the writer during this research. Some of the published papers were without bibliography and it is doubtful whether any serious attempt was made to investigate priority of publication, or to prevent duplication.

Many interesting papers have appeared in the literature on esophagoplasty but only those were referred to which seemed to contribute to the development of the various techniques. Those articles which represent duplications or similar procedures to previously described operations have been omitted purposely.

Most of the procedures mentioned are now obsolete but were links in the chain of development and are of historical interest only. In the United States, gastroesophagostomy after mobilization of the stomach is the operation of choice. In Europe, other types of esophagoplasty procedure are in use. Yudin has had a large experience in the use of the jejunum in esophagoplasty.

In certain instances some of the little used methods of esophagoplasty may have advantage over the more commonly employed methods, and a knowledge of these methods is valuable when the usual techniques do not seem adequate.

REFERENCES

Abbe, R.: Esophageal Stricture Cured by Cutting with a String. *Ann. Surg.*, 19: 88–89, 1894.

Ach: Beitrag zur Oesophaguschirugie. *Müchen med. Wchschr.*, 60: 1115, 1913.

Adams, W. E., and D. B. Phemister: Carcinoma of Lower Throacic Esophagus. Report of a Successful Resection and Esophagogastrostomy. *J. Thoracic Surg.*, 7: 605–620, 1938.

Allen, Duff: Further Experimental Reconstruction of Esophagus with Autogenous Fascia Lata Transplants. *Arch. Surg.*, 10: 374–391, 1925.

Allen, D. S.: Experimental Reconstruction of Esophagus with Autogenous Fascia Lata Transplants. *Ann. Surg.*, 76: 157–175, 1922.

Allison, P. R.: Oesophago-jejunostomy for Irremovable Carcinoma of the Cardia. *Thorax*, 1: 239–246, 1946.

Beck, C., and A. Carrell: Demonstration of Specimens Illustrating a Method of Formation of a Prethoracic Esophagus. *Illinois M.J.*, 7: 463–464, 1905.

Biondi, D.: Esofago-gastrostomia sperimentale intratoracica. *Supplemento al Policlinico*, 1: 964, 1895.

Bircher, Eugen: Ein Beitrag zur plastischer Bildung eines neues Esophagus. *Zentralbl. f. Chir.*, 34: 1479–1482, 1907.

Blauel: Zur totalen Oesophagoplastik. *Beitr. z. klin. Chir.*, 104: 46–79, 1916.

Carter, B. N.: Resection of a Portion of the Thoracic Esophagus for Carcinoma. *Surg., Gynec. and Obst.*, 71: 624–632, 1940.

Christophe, L.: Fistule oesophagienne dans un pneumothorax extrapleural droit; oesophagoplastie sous-cutanee aux depens du colon transverse. *Acta chir. belg.*, 50: 127–131, 1951.

Davis, J. S., and E. S. Stafford: Successful Construction of an Extra-thoracic Esophagus. *Bull. Johns Hopkins Hosp.*, 71: 191, 1942.

Dobromysslow, W. D.: Ein Fall von transpleuraler Oesophogotomie in Brustabschnitte d. Speiseröhre. *Zentralbl. f. Chir.*, 28: 18, 1901.

Dinnick, Tilson: The Origins and Evolution of Colostomy. *Brit. J. Surg.*, 22: 142–154, 1934.

Eggers, Carl: Resection of Thoracic Portion of Esophagus for Carcinoma; Report of a Successful Case. *Arch. Surg.*, 10: 361–373, 1925.

Engelbrecht, H.: Beitrag zur antethorakalen Oesophagoplastik. *Beitr. z. klin. Chir.*, 126: 277–286, 1922.

Fink, Franz: Ueber plastischen Ersatz der Speiseröhre. *Zentralbl. f. Chir.*, 40: 545–547, 1913.

Fischer, Herman: Surgical Treatment of the Esophagus. *Arch. Surg.*, 6: 256–273, 1923.

Frangenheim, Paul: Oesophagoplastik. *Ergebn. de Chir. u. Orthop.*, 5: 407–431, 1913.

Fry, Wesley: Experimental Intrapleural Substitution of the Right Colon for the Resected Esophagus, *Surg., Gynec. and Obst*, 96: 315–322, 1953.

Galpern, J.: Eine neue Methode der Oesophagoplastik. *Zentralbl. f. Chir.* 52: 182–184, 1925.

Garlock, J. H.: Re-establishment of Esophagogastric Continuity Following Resection of Esophagus for Carcinoma of the Middle Third. *Surg., Gynec. and Obst.*, 78: 23–28, 1944.

————: Surgical Treatment of Carcinoma of the Esophagus. *Arch. Surg.*, 41: 1184–1214, 1940.

————: The Surgical Treatment of Carcinoma of Thoracic Esophagus with a Report of Three Successful Cases. *Surg., Gynec. and Obst.*, 66: 534–548, 1938.

Gosset, A.: De L'oesophago-gastrostomie trans-diaphragmatique. *Rev. Chir.*, 12: 694–707, 1903.

Green, N. W.: A Positive Pressure Method of Artificial Respiration with a Practical Device for Its Application in Thoracic Surgery, together with a Report of Transpleural Operations on 14 Dogs. *Surg., Gynec. and Obst.*, 2: 512–527, 1906.

Hacker, von: Ueber Oesophagoplastik in allegemeinen und über den Ersatz der Speiseröhre durch antethorakal Hautdickdarmschlauchbildung in besonderen. *Deutsche med. Wchnschr.*, 40: 991–992, 1914.

Hanrahan, M.: Use of Migrated Skin Flaps for Both Lining an dCovering Artificial Antethoracic Esophagus. *Plast. and Reconstruct. Surg.*, 2: 474–480, 1947.

Herzen, Peter: Ein Modifikation der Rouxschen Oesophago-jejunogastrostomie. *Zentralbl. f. Chir.*, 35: 219–222, 1908.

Hushang, Javid: Bridging of Esophageal Defects with Fresh and Preserved Aorta Grafts, Reported at 38th Clinical Congress of the American College of Surgeons, September 22, 1952.

Jiano, J.: Contribution a l'oesophagoplastie prethoracique. *Arch. ital. chir.*, 52: 580–587, 1938.

Jianu, Amza: Gastrostomie and Oesophagoplastik. *Deutsche Ztschr. f. Chir.*, 118: 383–390, 1912.

Jones, S.: Gastrostomy for Stricture (cancerous) of Esophagus; Death from Bronchitis Forty Days after Operation. *Lancet*, 1: 678–679, 1875.

Kay, Earle B.: Experimental Observations on Reconstructive Intrathoracic Esophagogastric Anastomosis following Resection of the Esophagus for Carcinoma. *Surg., Gynec. and Obst.*, 76: 300–314, 1943.

Kelling, G.: Zur Frage der intrathorokalen Operation der Speiseröhre. *Zentralbl. f. Chir.*, 31: 609, 1904.

————: Oesophagoplastik mit Hilfe des Quercolon. *Zentralbl. f. Chir.*, 38: 1209–1212, 1911.

Keyes, T. E.: *History of Surgical Anesthesia.* New York City, Schuman's, 1945.

Kirschner: Ein neues Verfahren der Oesophagoplastik. *Arch. f. kin, Chir.*, 114: 606–663, 1920.

Kresmen, A. J.: A Combined Abdominothoracic Incision Particularly Adapted for Use in Total Gastrectomy and Esophagogastrectomy. *Surgery*, 24: 605–612, 1948.

Kummel, Weber: Intrathorakel Oesophagoplasty. *Beitr. z. klin. Chir.*, 126: 264–277, 1922.

Levy: Versuche über die Resektion der Speiseröhre. *Arch. f. klin. Chir.*, 56: 839, 1898.

Lexer, E.: Oesophagoplastik. *Deutsche med. Wchnschr.*, 58: 1548–1550, 1911.

Longmire, W. P., Jr.: Modification of Roux Technic for Antethoracic Esophageal Reconstruction; Anastomosis of Mesenteric and Internal Mammary Blood Vessels. *Surgery*, 22: 94–100, 1947.

Longmire, W. P., Jr., and M. M. Ravitch: A New Method for Constructing an Artificial Esophagus. *Ann. Surg.*, 123: 819–835, 1946.

Mes, G. M.: New Method of Oesophagoplasty. *J. Internat. Coll. Surgeons*, 11: 270–277, 1948.

Meyer, Willy: Esophagogastrostomy after Intrathoracic Resection of the Esophagus. *Ann. Surg.*, 1: 175–189, 1909.

Miscall, L., and B. B. Clerk: Murphy Button in Esophagogastrostomy. *Surgery*, 14: 83–87, 1943.

Murphy, Fred T.: A Suggestion for a Practical Apparatus for Use in Intrathoracic Operations. *Boston Med. and Surg. J.*, 152: 428–431, 1905.

Nasiloff: Oesophagotomie et resection endothoracica. *Vrach.*, 9: 481, 1888, cited by Fischer (1923).

Neuhof, H.: Experimental Reconstruction of Esophagus by Granulation Tubes. *Surg., Gynec. and Obst.*, 34: 767–775, 1922.

Nicolaysen, J.: Antethorakel Oesofagoplastik. *Norsk. mag. f. laegevidensk.*, 85: 905–928, 1924.

Ochsner, Alton and DeBakey, Michael: Surgical Aspects of Carcinoma of the Esophagus. *J. Thoracic. Surg.*, 10: 401–445, 1941.

Orsoni, Paul, et Marcel Lemaire: Technique des oesophagoplasties par le colon transverse et descendant. *J. de chir.*, 67: 491–505, 1951.

Rapin, M.: L'oesophagoplastie prethoracique par un procede nouveau et esthetique au moyen de la greffe epitheliale incluse. *Helvet. med. acta*, 8: 429–435, 1941.

Razzaboni, G.: Intestinal Graft in Esophagus. *Policlinico (sez. chir.)*. 26: 1, 1919.

Rehn, Edward: Oesophagus. *Chir. Jena*, G. Fisher, 1914, cited by Fischer (1923).

Reisenkampf, O.: Ein Fall von totalen Oesophagoplastik. *Deutsche Ztschr. f. Chir.*, 193: 116–119, 1925.

Reynolds, J. T., and Young, J. P.: Use of Roux-Y in Extending Operability of Carcinoma of Stomach and of Lower End of Esophagus. *Surgery*, 24: 246–263, 1948.

Reinhoff, W. F., Jr.: Intrathoracic Esophagojejunostomy for Lesions of the Upper Third of the Esophagus. *South. M.J.* 39: 928–940, 1946.

Robertson, C. W., C. W. Howe, and R. H. Smithwick: Use of Colon to Replace Lower Esophagus in Man: Report of a Case Four Years after Operation, Reported at 38th Clinical Congress of the American College of Surgeons, September 22, 1952.

Roith, O.: Antethorakal Oesophagus. *Deutsche Ztschr. f. Chir.*, 183: 419–423, 1924.

Roux, Cesar: L'oesophago-jejunogastrostomose nouvelle operation pour retrecissement infranchissable de l'oesophage. *Semaine med.*, 27: 37–40, 1907.

Rovsing, T.: Antethoracic Oesophagoplasty. *Ann. Surg.*, 81: 52–58, 1925.

Rudler, J. C.: Enquete Chirurgicale. *Rev. de chir.*, 29: 193–205, 1951.

Rudolf, A.: Totale Oesophagoplastik aus dem Dickdarm. *Beitr. z. klin. Chir.*, 147: 53–55, 1929.

Sauerbruch, Ferd.: Die Anastomose zwischen Magen und Speiseröhre und die Resetion des Brustabschnittes der Speiseröhre. *Zentralbl. f. Chir.*, 32: 81–86, 1905.

————: Bericht über die ersten in der pneumatischen Kammer der Breslauer Klinik ausgefuhrten operationen. *München med. Wchnschr.*, 53: 1–4, 1906.

Sweet, R. H.: Surgical Management of Carcinoma of Middle Third of Esophagus; Preliminary Report. *New England J. Med.*, 233: 1–7, 1945.

————: Transthoracic Gastrectomy and Esophagectomy for Carcinoma of the Stomach and Esophagus. *Clinics*, 3: 1288–1315, 1945.

————: Gastrostomy in Cases of Carcinoma of the Esophagus. *Surg., Gynec. and Obst.*, 73: 55–62, 1941.

Swenson, O.: Anterior Thoracic Esophagoplasty. *Am. J. Surg.*, 74: 69–79, 1947.

Tavel, E.: Eine neue Methode der Gastrostomie. *Zentralbl. f. Chir.*, 33: 634–635, 1906.

Torek, Franz: The First Successful Case of Resection of the Thoracic Portion of the Esophagus for Carcinoma. *Surg., Gynec. and Obst.*, 13: 614–617, 1913.

————: Carcinoma of Thoracic Portion of Esophagus; Report of a Case in which Operation was Done 11 Years ago. *Arch. Surg.*, 10: 353–360, 1925.

Turner, G. G.: Excision of Thoracic Esophagus for Carcinoma, with Construction of Extrathoracic Gullet. *New England J. Med.*, 210: 947–948, 1934.

Voelcker: Ueber Extirpation der Kardia wegen Karzinom. *Deutsche Gesellsch. f. Chir.*, 1: 126–129, 1908.

Vulliet, Henri: De l'esophagoplastie et des diverses modifications. *Semaine med.*, 31: 529–530, 1911.

Wullstein, L.: Ueber antethroakale Oesophago-Jejunostomie und Operationen nach gleichem Prinzip. *Deutche med. Wchnschr.*, 30: 734–736, 1904.

Yudin, S. S.: The Surgical Construction of 80 Cases of Artificial Esophagus. *Surg., Genec. and Obst.*, 78: 561–583, 1944.

Zaaijer, J. H., Erfolgreiche transpleurale Resektion eines Kardiakazinomes. *Beitr. z. klin. Chir.*, 83: 419–430, 1913.

OBSERVATIONS ON THE BEHAVIOR OF EXFOLIATED CELLS FROM BREASTS OF DOGS IN TISSUE CULTURE*

W. C. McCormick, Dudley Jackson, and C. M. Pomerat

Introduction. For more than a decade one of us (D. J.) has been particularly concerned with the cytological analysis of breast secretions as a clinical aid both for diagnostic purposes and to follow the natural history of breast pathology in patients who could be kept under observation for a number of years. The findings resulting from this work have been summarized by Jackson and Severance (1946) and by Jackson, Todd, and Gorsuch (1951).

Pathological conditions of the breast often associated with breast secretion are intraductal papilloma, chronic cystic mastitis and intraductal carcinomas; in fact, nipple discharge is the most frequent symptom of the patient with papilloma of the breast (Haagensen, Stout, and Phillips, 1951). In 50 per cent of patients with papilloma a discharge is the only symptom. Observations on human subjects have led Jackson, Todd, and Gorsuch (1951) and Copeland and Geschister (1950) to believe that the so-called "silent papilloma" may be a premalignant lesion while Haagensen, Stout and Phillips assert with equal vehemence that it is definitely benign.

Aided by grants from the National Cancer Institute and the Pan-American Cancer Foundation a team of workers consisting of Drs. D. Jackson and D. A. Todd of San Antonio, C. M. Pomerat and W. C. McCormick of The University of Texas Medical Branch at Galveston, and T. Sergeant and J. A. Fauver at Trinity University in San Antonio, undertook the problem of trying to resolve this important question with a cytological study of cells from breast secretion in tissue culture. The first basic step in this study was to prove that exfoliated cells from the breast would grow in tissue culture. This has been established as a feasible objective (*c.f.* Pomerat, 1952).

---

* From the Tissue Culture Laboratory, The University of Texas Medical Branch, Galveston, and the Nix Hospital, San Antonio. Aided by grants from the National Cancer Institute and the Pan-American Cancer Foundation. Received for publication January 5, 1953.

By virtue of the wide spectrum of breast pathology found in female dogs and the multiple breasts containing different degrees of pathology, this species is being used as the laboratory animal of choice for critical experimental studies which are being conducted with reference to observations made upon the human female.

The object of the present paper consists of a more detailed study of the events observed when dog breast secretions are grown by the tissue culture method and also to present preliminary findings on the effects of steroid hormones on these same secretions in tissue culture.

Materials and Methods. Three old bitches were selected from the city pound who showed evidence of breast pathology as revealed by the secretions collected from their nipple ducts. These dogs were not lactating. Since the main object of the work was not to study dogs with carcinomas but to make observations with "silent papilloma" the animals were also chosen on the basis of absence of palpable tumors. The dogs were old and poorly nourished but after feeding them a high protein diet they quickly returned to a good nutritional status. The character of the breast pathology as revealed by the breast secretion is given in Table 1.

Secretion was expressed from the breast by gentle pressure, beginning at the periphery of the breast and gradually working toward the tip of the nipple. The secretion was collected in a small pipette with a rubber bulb. No attempt was made to separate the secretion from the various ducts. Breasts were numbered serially in a cranio-caudal direction for the right and left sides.

Tissue cultures were set up immediately after the secretion was obtained. Two drops of homologous serum were placed on cover glasses measuring 12 x 50 mm. and of No. 1 thinness. To this one drop of the breast secretion was added. A clot was formed by adding two drops of heparinized cockerel plasma and two drops of tissue extract made from 7–8 day chick embryos. The cover slips were transferred to 15 x 150 mm. Pyrex test tubes and after allowing the clot to set for 1–2 hours, two ml. of fluid nutrient consisting of 50 per cent Gey's balanced salt solution, 45 per cent human ascitic fluid and 5 per cent embryonic extract together with a final concentration of 1,000 units of sodium penicillin G per ml. were added. The cultures were then placed in a drum in nearly horizontal position, the drum making 8 revolutions per hour. The fluid medium was changed every third day.

Direct observations through the test tube with a 32 mm. objective and a 10 x ocular were made to ascertain the general character of the outgrowth. For more detailed study the cover slips were removed from the tubes, mounted on supports made with strips of paraffin on a glass slide and then sealed with paraffin. These were used for study with the 8 and 1.8 mm.

phase objectives. Photomicrographs were made with such preparations. Cinematographic records were obtained with the use of a time lapse device (Rolab Co.) at a rate of 1 and 8 frames per minute. A Bausch and Lomb ribbon filament lamp was used with water, heat absorbing glass and one or more neutral density filters as well as a Wratten B 58 green filter. For the study of the effects of sterols on the cultures with the use of phase microscopy and time lapse cinematography, the cover slips were set up in special perfusion chambers (Pomerat, 1951). The sterols used in the experiments were obtained from Ayerst, McKenna, and Harrison. They were sodium estrone sulfate with 60 per cent disodium-phosphate and sodium testosterone sulfate, both being water soluble preparations. The sterols were dissolved in a small amount of Gey's solution and sterilized by passage through a Seitz filter. The sterols in Gey's solution were made up to twice the desired concentration since they made up half of the volume of the fluid nutrient. No attempt was made to incorporate the sterols into the plasma clot since the material was known to be readily soluble.

Hematoxylin-eosin-azure, following Helly Zenker fixation, was used to stain initial smears. For staining routine cultures after fixation in absolute methyl alcohol, the method of Jacobson (1952) (a combination of May-Grunwald and Giemsa) was employed. For fat staining, Sudan Black B with hematoxylin was used. A mitochondria stain was used in which the cultures were fixed in iodine vapor followed by Bensley's solution (2 parts of 2 per cent osmium tetroxide and 8 parts of 3 per cent potassium dichromate) and treated with Heidenhain's hematoxylin.

In order to demonstrate characteristics of the outgrowth of breast tissue *in vitro* three breasts from Dog No. 3 were biopsied. At the time pieces of tissue from each breast were fixed in formalin for sectioning. The breasts biopsied were L_3, R_2, and L_1 with a diagnosis by smear technique as shown in Table 1.

The tissue culture preparations were made in the same way as for the breast fluid except three small pieces of tissue, about 1 x 1 mm., were placed on the cover slip instead of the breast fluid.

In sectioning, the tissues were imbedded and a few sections cut for L_3 and R_2. In case of L_1 one-half the breast was imbedded and multiple sections taken, examining very carefully that tissue near the large ducts. For staining hematoxylin-eosin, Jacobson, and iron hematoxylin were used.

Observations. Breast secretions were obtained once or twice a week from 21 nipples of the 3 dogs. In Dogs No. 1 and No. 2 this fluid soon disappeared. All nipples on these two dogs became dry in 3–8 weeks. The exact cause of this change is unknown. It is possible that the dogs were first used a short time after lactation and a residuum of milk secretion remained. Also it will

TABLE 1

Breast Secretion

Dog and nipple numbers	Amount of secretion	Characteristic of secretion	Cell types	Diagnosis of smears by Todd s classification
1—R 3.....	Slight	Pearly	Few degenerating polymorphonuclear cells; ductal epithelial cells.	Class II
L 1.....	Slight	Clear and pearly	Ductal epithelial cells, clumps of degenerating polymorphs.	Class II
L 4.....	Several drops	Bloody	RBC, polymorphs, ductal epithelial cells and pseudo clusters.	Class III-C
2—R 2.....	Scant	Cloudy	Occasional degenerating polymorphs and ductal epithelial cells.	Class II
R 4.....	Scant	Cloudy	Occasional degenerating cell.	Class I
R 5.....	Scant	Cloudy	Few ductal epithelial cells.	Class I
L 2.....	Scant	Cloudy	Occasional degenerating cell.	Class I
L 3.....	Scant	Cloudy	Ductal epithelial cells, few phagocytes.	Class II
L 4.....	Scant	Cloudy	Occasional phagocyte, few ductal epithelials and pseudo clusters.	Class III-B
L 5.....	Several drops	Bloody	Many RBC, polymorphs, ductal epithelial cells and cell clusters.	Class IV-A

3—R 1.....	Scant	Pearly-gray	Few polymorphonuclear cells and secretion cells.	Class I
R 2.....	Plentiful	Pearly-gray and serous	Few polymorphonuclear and ductal epithelial cells and clusters.	Class IV-B
R 3.....	Plentiful	Serous	Polymorphonuclear cells, ductal epithelial cells and clusters.	Class IV (III-B)
R 4.....	Plentiful	Serous	Few cells—ductal epithelial cells, hyperplasia.	Class II
R 5.....	Plentiful	Bloody	Degenerating RBC, polymorphonuclear cells, ductal epithelial cells and clusters.	Class IV (III-A)
L 1.....	Scant	Serous	Polymorphonuclear cells and ductal epithelial cells.	Class I
L 2.....	Scant	Serous	Polymorphonuclear cells and ductal epithelial cells.	Class II
L 3.....	Profuse	Pearly-gray	RBC and ductal epithelial cells and clusters.	Class III
L 4.....	Plentiful	Bloody	RBC and ductal epithelial cells and clusters.	Class IV-B
L 5.....	Plentiful	Bloody	RBC and ductal epithelial cells and clusters.	Class IV-A

Todd's Classification

Class I—Normal.
Class II—Hyperplasia.
Class III—Doubtful positive.
Class III-A—With red blood cells.
Class III-B—Without red blood cells.
Class III-C—With many red blood cells.
Class IV—Positive for papilloma.
Class IV-A—With red blood cells.
Class IV-B—Without red blood cells.
Class V—Carcinoma.

be noted that most of these breasts showed only epithelial hyperplasia; that is, epithelial-like cells not arranged in clusters. This could have been due to physiological changes or because the residual secretion expressed was not replaced in the nipple ducts. In Dog No. 3, on the other hand, where most of the fluid showed cells arranged in clusters, the same schedule was followed and the secretion from 6 of the breasts had remained free flowing during more than 12 weeks.

1. *Direct Smears.* The fluid and cell types encountered in the breast secretion had the characteristics shown in Table 1. Although in some nipples the fluid disappeared, there was no change in the general character of the cell types in these and in Dog No. 3 during the period of observation. Direct smears were stained only at 3–4 week intervals. It is possible that over a longer period some of the secretion with only hyperplasia or doubtful clusters might have been transformed into true papillomatous clusters.

In general, the initial smears had a few degenerating polymorphonuclear cells while some showed degenerating red blood cells. In figure 1, however, a dense collection of isolated cells are shown together with one cell cluster. All of the secretions had cells which were very large (20–50 microns in diameter), round, having a distinct cell membrane, foamy cytoplasm with granules and vacuoles and a central or eccentric faint-staining nucleus (Fig. 1). These elements were thought to be desquamated ductal epithelial cells—the so-called "colostrum-like cell" of Cheatle and Cutler (1931). However, Maximow and Bloom (1948) believe that they are more likely to be wandering lymphoid cells which escape into the glandular spaces.

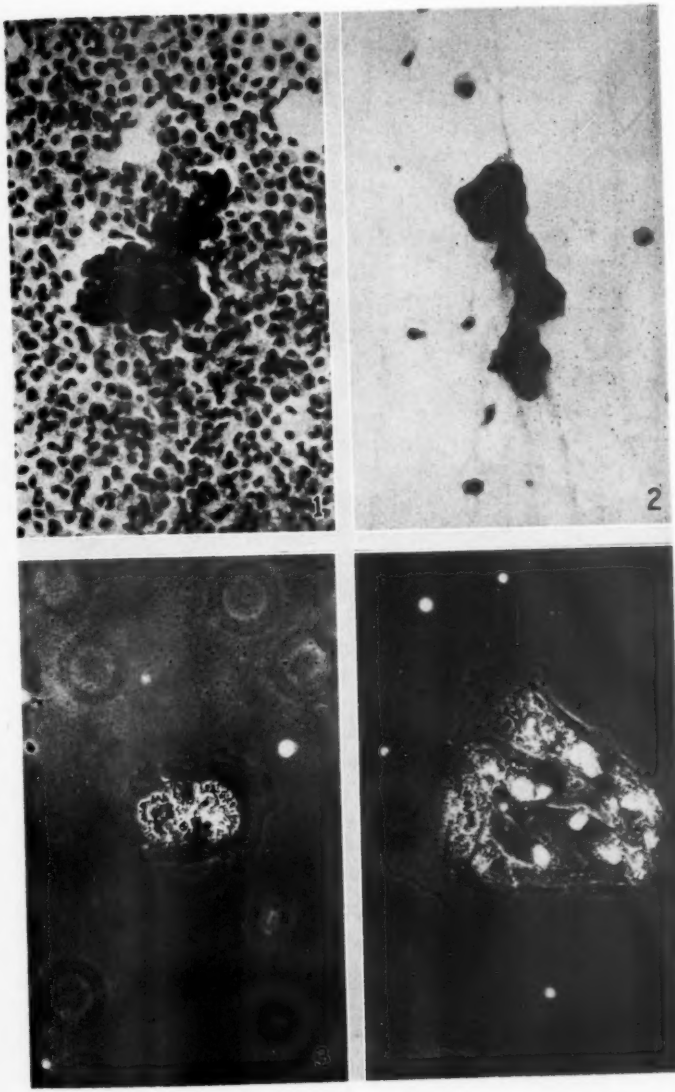
PLATE I

FIGS. 1–2. Direct smears obtained by Jacobson's method following fixation in absolute methyl alcohol. Magnification approximately 500 x.

FIG. 1. Initial smear from Dog No. 2 showing: (a) Degenerating polymorphonuclear cells, (b) macrophages, and (c) an epithelial cluster.

FIG. 2. Initial smear from Dog No. 3 showing a typical cell cluster of the papillomatous type (Class IV).

FIGS. 3–4. Dark phase contrast photomicrography of emigrating epithelial elements from a 4-day culture of exfoliated cells in breast secretion. Note the absence of clear cell boundaries, the peripheral extension of active cytoplasm, the prominent single central nucleolus and the presence of numerous refractile perinuclear granules. Approximately 500 x.


2

Epithelial clusters were arranged in clumps of 2–30 or more cells (Fig. 1). A papillomatous cluster is shown in figure 2. Individual elements were irregular in shape but tended to be smaller than the isolated ductal epithelial or "colostrum-like cell." The cell membranes were not as distinct, the cytoplasm was not as foamy and vacuolated as that of the other cells present. The nuclei were much darker after staining and often elliptical or oval rather than round; some cells were binucleate. Most of the nuclei contained two distinct nucleoli which were best seen in the living state with phase contrast microscopy after 1–2 days in tissue culture.

2. *Tissue Culture.* Some of the tissue cultures were observed with phase contrast objectives immediately after the clot had been formed and the fluid nutrient had been added. The clusters here showed rounded cells similar to those of the initial smears. Even at this time the cells showed granules in active movement. Time lapse cinematographic records revealed that by the end of 4 hours the cell membranes had become extended, the cells flattened and emigration of spindle cells on the cover glass was initiated. Within 12 hours the cell clusters had become arranged in typical epithelial sheets with membrane activity characteristic of well established cultures.

In Dog No. 1 cells from only one breast, L_4, showed outgrowth. By the 3rd day there were a few clusters. As a whole, the epithelial clusters grown in tissue culture resemble papillary processes as seen in sections of fixed tissue except that they were more flattened and tended to spread out more than in the sections, thus making the cells appear larger and the cytoplasm more plentiful (Figs. 3–8). In addition to the epithelial clusters, another interesting feature of this breast was that the isolated ductal epithelial cells had become changed morphologically. The

---

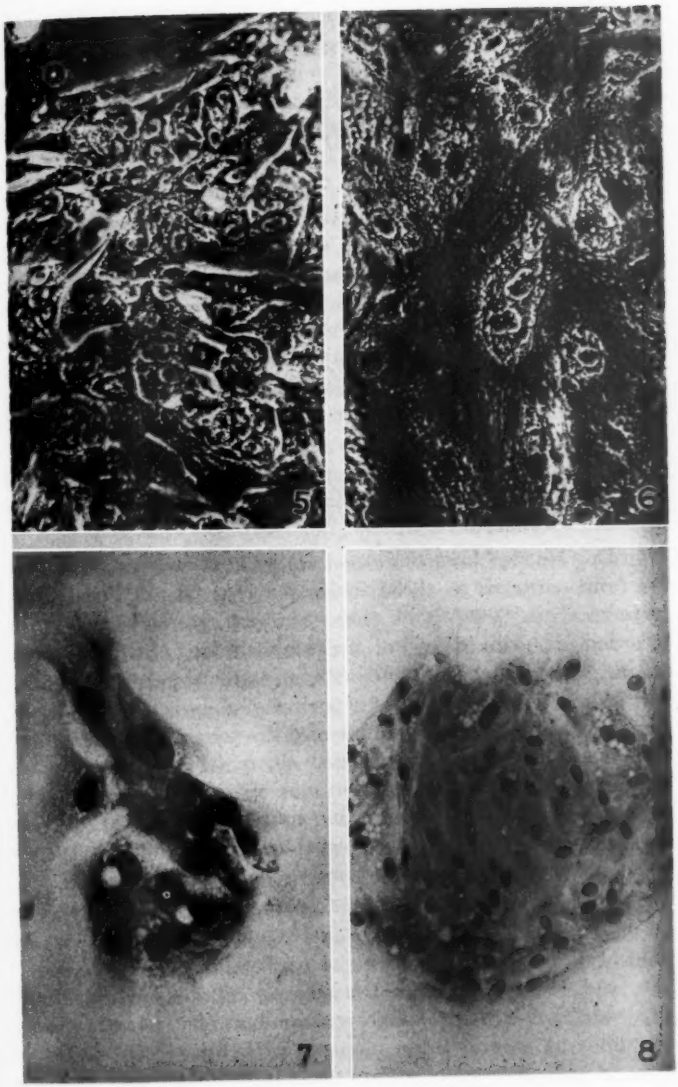
PLATE II

FIGS. 5–6. Dark phase contrast photomicrography of living exfoliated cells from breast secretion. Magnification approximately 500 x.

FIG. 5. Margin of an epithelial sheet developed after 2 days in culture which was probably derived from a papillomatous cluster.

FIG. 6. Central area of an epithelial sheet 9 days in tissue culture. A large binucleate cell with well-defined cell boundaries in the center of the photograph.

FIGS. 7–8. Exfoliated cells in tissue culture stained by Jacobson's method, following absolute methyl alcohol fixation on the 6th day in tissue culture, showing characteristic nuclei and vacuolar spaces in the cytoplasm. Magnifications: figure 7, 750 x; figure 8, 500 x.


5
6
7
8

nuclei increased in size, became strongly basophilic, were generally eccentric and the cytoplasm appeared as if it was streaming behind the nuclei. There was no evidence of mitosis in these cells but some of them showed amitotic patterns, so the question arose as to whether they represented true epithelial cells capable of further growth in tissue culture.

In Dog No. 2, two breasts, L_4 and L_5, had cells become established in culture. From these breasts approximately 200 feet of time lapse movies were taken with phase contrast objectives. Of particular interest in these records were the very active membranes showing cell drinking (pinocytosis). The membranes seemed to have a definite rhythmic pattern in their movement. The small clusters were actively motile and over a few hours' time moved out of the optical field. Some of the living cells have been multinucleate. This was found to be true in stained clusters. However, there was no achromasia, hyperchromicity or bizarre arrangement suggestive of a malignant process. In these cells the nuclei were quite distinct, of about equal size and the cytoplasm stained very faintly.

In Dog No. 3, 7 breasts showed outgrowth *in vitro*. These gave the same patterns as those found for Dog No. 2. From these cultures about 500 feet of phase contrast cinematography was recorded. The life history of the establishment of colonies from the minute the clot was formed and the supernatant added was followed with the use of a perfusion chamber. Some injury from the microscope lamp influenced the course of the evolution of epithelial sheets from rounded clusters but a general idea of the method of transformation was obtained. The activity of filamentous mitochondria in the epithelial cells was also recorded with time-lapse cinematography and oil immersion phase contrast objectives.

3. *Hormone Studies* (Preliminary studies). Using perfusion chambers, cultures incubated for 4 days were treated with 48 γ/ml. sodium estrogen sulfate, 24 γ/ml. of sodium estrone sulfate, and 48 γ/ml. sodium testosterone sulfate and the effects were recorded with time-lapse cinematography. Membrane retraction was observed the moment a perfusate containing 48 γ/ml. of sodium estrone sulfate came into contact with the cells and by the end of 2 hours they appeared almost immobile, with

marked cytoplasmic retraction but on addition of the fluid nutrient medium without the hormone the cluster quickly re-assumed its initial morphological appearance and activity. After a short period of this perfusion, 24 γ/ml. sodium estrone sulfate was introduced into the culture chamber. With this concentration no marked obvious change in the epithelial cluster could be noted although there seemed to be a slight withdrawal of the membranes on the side from which the perfusate was coming. This was washed out with the basic nutrient and 48 γ/ml. of sodium testosterone sulfate was introduced in the perfusate. This concentration of the hormone seemed to stimulate the cells to greater activity. All of these observations were made on the same cluster of cells. The study extended over a 24-hour period. At the end of this time the sodium testosterone sulfate was washed out and the cluster regained its original appearance and behavior.

The other method of studying the effect of the hormones was to add them to the fluid nutrient and allow them to remain in contact with the cells for several days or by adding it the night before changing the fluid nutrient so that only intermittent stimulation might occur. After 4–6 days in tissue culture with continuous contact with 33 γ/ml. sodium estrone sulfate there was a very marked increase in the number of granules in the cytoplasm. In most instances these were of a very uniform size of about 1 micron and formed a compact pattern in the cytoplasm giving a honeycomb effect. The outer margin of the active cytoplasmic membrane was completely devoid of these spherules (Figs. 9–13). The number of granules appeared to be partly a function of time, their number increasing until the 8th to 10th day when a maximum was reached. The cultures receiving intermittent treatment with hormone had less than those under continuous contact. Besides static photomicrographs, phase contrast cinematographic records were obtained of cultures chronically treated with hormones but it was found that their cells were very sensitive to light so that many of them quickly rounded up and died during the process of photography.

Hormone treated cultures were stained with Sudan Black B and the granules were found to be sudanophilic (Figs. 12–13). A record of the cluster was obtained with phase contrast micro-

scopy, then the culture was perfused with Sudan Black B and another photomicrograph was taken. This was followed by an ordinary light photograph to show the granules with the Sudan Black. This work is of a preliminary nature and is introduced here merely as a suggestion for future work.

4. *Biopsy Material.* In tissue culture there was an excellent fibroblastic outgrowth from explants of all three breasts. Many mitoses were observed in these cells (Figs. 14 and 15). Islands of epithelial cells and small epithelial sheets were demonstrated in cultures from R_2 and L_4. Breast L_3 with a diagnosis of doubtful positive for papilloma (Table 1) had no epithelial sheets.

As noted previously none of the breasts had palpable tumors. The few sections available for study from R_2 and L_3 showed ducts filled with epithelial cells but no papilloma was demonstrated. From breast L_4 a multi-radicular papillary outgrowth was present near the nipple (Figs. 16 and 17).

Discussion. The main objective of this report has been to demonstrate that exfoliated cells from the nipples of dogs with breast pathology would "grow" in tissue culture. This "growth" seems to be a combination of true mitosis, amitosis and emigration of cells from densely packed clusters to form flattened expanded colonies. We must assume that all of the growth cannot be due to direct division since the number of cells in mitosis by the second day is not great enough to account for the size of the colony. There was no evidence of complete division by amitosis, but two daughter nuclei were often found in the same cell which leads one to suspect amitotic activity.

The question of which cells will grow in tissue culture also arises. Typical epithelial sheets probably arise from pre-exist-

---

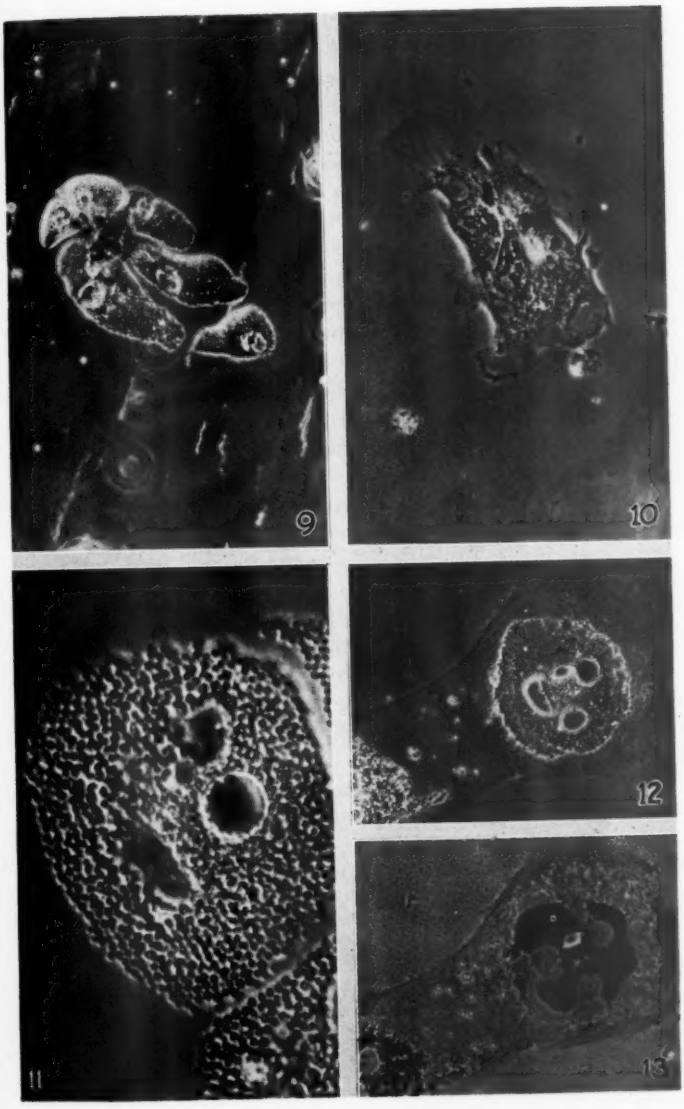
PLATE III

FIGS. 9–13. Dark phase contrast photomicrography of exfoliated cells from breast secretion in tissue culture. Magnification approximately 500 x, except figure 11 which was 2340 x.

FIGS. 9–10. An epithelial sheet showing a massive accumulation of cytoplasmic granules of very uniform size and distribution in a culture treated with 33 of sodium estrone sulfate (Ayerst, McKenna, and Harrison) for 10 days (Fig. 9) as compared with an untreated control.

FIG. 11. A cell similar to those shown in figure 9 but at a magnification of approximately 2340 x.

FIGS. 12–13. Accumulation of droplets in cells treated for 6 days, like those shown in figure 9. Figure 12 was made before and figure 13 about 10 minutes after treatment with Sudan Black B.


9
10
11
12
13

ing epithelial clusters. However, in comparing initial smears and tissue cultures preparations that had the same amount of breast secretion present, more epithelial clusters are present in the tissue cultures than can be accounted for by "growth" of true papillomatous clusters. From this observation we must infer that so-called pseudo clusters or doubtful clusters will give rise to epithelial sheets by tissue culture methods. No epithelial sheets were observed in cultures from breasts that were consistently diagnosed as Class I and II (Table 1) by the smear technique. Cultures showed occasional macrophages which probably came directly from the explanted cell population and continued to live for a few days. Spindle cells of mesothelial origin were observed but we did not trace their origin and evolution. The ductal epithelial cells seem to have been true epithelial cells or some type of histocytic element (Cheatle and Cutler, 1931; Maximow and Bloom, 1948). Saphir (1950) recognizes two types of macrophages and ductal epithelial cells; one type of macrophage with foamy cytoplasm, vesicular nucleus and no intracytoplasmic inclusions, another type with large usually peripheral nucleus which is hyperchromatic and intracytoplasmic globules probably of fatty nature. He describes the ductal epithelial cells as being of uniform shape, rarely single, with a large nucleus and distinct cell membranes. In tissue culture it has been observed that the exfoliated cells do not behave as typical macrophages which show extreme membrane activity and marked phagocytosis but these cells behave more like epithelial cells with slight mobility and little tendency to phagocytize as compared to typical macrophages, although they do show these properties. Also by time-lapse cinematography these cells have been followed until they unite with characteristic epithelial sheets, which indicates they are of epithelial origin.

PLATE IV

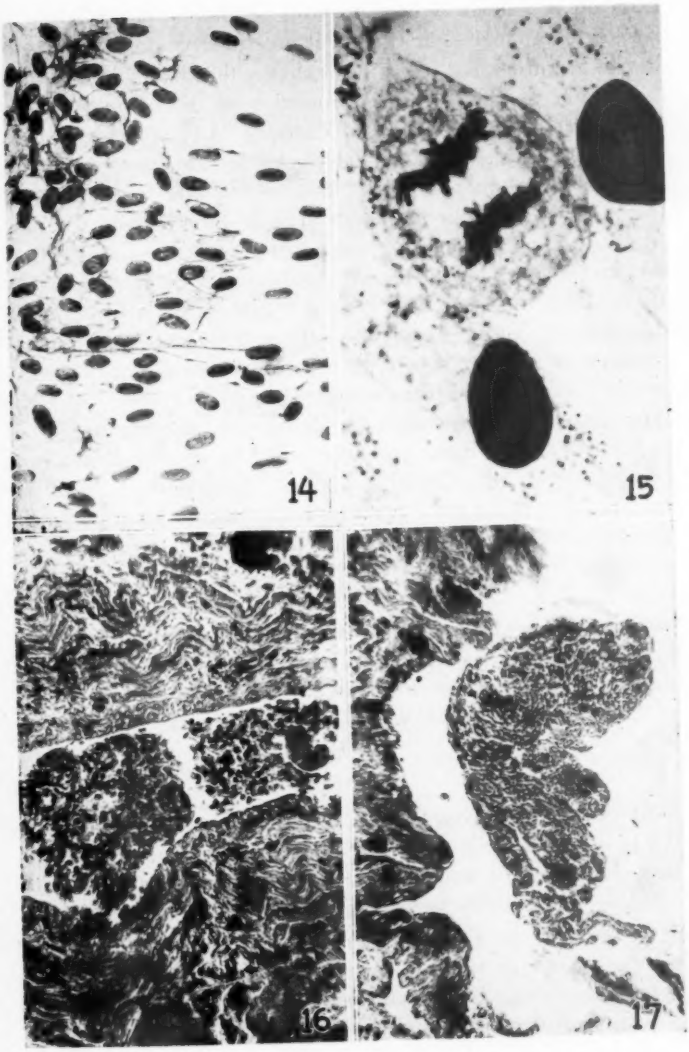
FIGS. 14–15. Outgrowth of cells from breast R_2 of Dog No. 3, stained by Jacobson's method following fixation on the 6th day in tissue culture.

FIG. 14. Typical fibroblasts at a magnification of approximately 500 x.

FIG. 15. Fibroblast in anaphase stage of mitoses, magnification 2340 x.

FIG. 16. Section through duct of breast L_4 of Dog No. 3, showing clumps of ductal epithelial cells within the duct. Jacobson's stain, magnification 500 x.

FIG. 17. Section of duct near nipple showing papillary cluster. From breast L_4 of Dog No. 3. Hematoxylin-Eosin stain, 500 x.


14
15
16
17

Data on the effect of hormones on papilloma of the breast in regard to cytological or tissue culture study could not be found in the literature but it has been shown that if a patient with a carcinoma of the breast is treated with hormones the malignant cells undergo certain changes. Among these are increased granularity of the cytoplasm, hyperchromicity of the nucleus and often pyknosis of the nucleus and degeneration of the cells. These observations are true in some instances but not in all (Adair, Mellors, Farrow, Escher, and Urban, 1949; Godwin and Escher, 1951; Herrman, Adair, and Woodward, 1947). In our observation on cells from a breast secretion treated with 33 γ/ml. of sodium estrone the only striking change noted during a period of 21 days was a very marked granularity. (Therapeutic blood levels of this hormone vary from 18–48 γ/ml.) The spherules in the cells were very uniform and sudanophilic. It is felt that the hormone probably is tied up in the cell in a lipid complex represented by the droplets. Another explanation for the presence of the fatty granules is that the estrogen may have stimulated the epithelial cells to secretory function which is represented by the granules. It is interesting to note that epithelium from the lung and ovary of the rat did not exhibit these granules when concentrations of sodium estrone sulfate as high as 80 γ/ml. were employed. The marked light sensitivity of these cells may have been due to a greatly increased absorption of light energy by the fatty droplets within the cell.

Conclusions

Exfoliated cells from the breasts of dogs have the characteristics of epithelial cells when grown in tissue culture. True papillary clusters and pseudo-clusters, aggregates of ductal epithelial cells, have the ability to form epithelial sheets in tissue culture.

Cultures of the exfoliated cells treated with sodium estrone sulfate exhibit marked, uniform sudanophilic granules in the cytoplasm, which have not been observed in other epithelial cells treated with estrogens.

The diagnosis of papilloma by smear technique was proven by biopsy in one case when no palpable tumor was present.

REFERENCES

Adair, F. E., R. C. Mellors, T. H. Farrow, G. C. Escher, and J. A. Urban: The use of estrogens and androgens in advanced mammary cancer; clinical and laboratory study of 105 female patients. *J.A.M.A.*, 140: 1193–1200, 1949.

Cheatle, G. L., and M. Cutler: Tumors of the breast. J. B. Lippincott Co., Philadelphia, 1931.

Copeland, M. M., and C. F. Gerschister: Diagnosis and treatment of premalignant lesions of the breast. *Surg. Clinic of N. Am.*, 30: 1717–1741, 1950.

Godwin, J. T., and G. C. Escher: Hormone-treated primary operable breast carcinoma; a pathological study of 33 cases. *Cancer*, 4: 136–140, 1951.

Haagensen, C. D., A. P. Stout, and J. S. Phillips: The papillary neoplasms of the breast. I. Benign intraductal papilloma. *Annals of Surg.*, 133: 18–36, 1951.

Herrman, J. B., F. E. Adair, and H. Q. Woodward: Effect of estrogenic hormone on advanced carcinoma of female breast. *Arch. of Surg.*, 54: 1–9, 1947.

Jackson, D., and A. O. Severance: Cytological study of nipple secretions: An aid in the diagnosis of breast lesions. *Texas State J. Med.*, 41: 512–515, 1946.

Jackson, D., D. A. Todd, and P. L. Gorsuch: Study of breast secretion for detection of intramammary pathologic change and of silent papilloma. *J. Intl. Coll. Surg.*, 15: 552–568, 1951.

Maximow, A. A., and W. Bloom: *Textbook of Histology*. 5th Ed., W. B. Saunders Co., Philadelphia, pp. 599, 1948.

Pomerat, C. M.: Tissue Culture Methods in *Methods in Medical Research*, Vol. IV, Year Book Publishers, Chicago, pp. 275–277, 1951.

————: Motion picture studies of living papilloma of the breast and breast cancer. *Texas Rep. Biol. and Med.*, 10: 217–227, 1952.

Saphir, O.: Cytological examination of breast secretions. *Am. J. Clin. Path.*, 20: 1001–1020, 1950.

EFFECTS OF INCREASING DOSES OF MORPHINE, DROMORAN AND SODIUM AMYTAL ON RAT TISSUES *IN VIVO* AND *IN VITRO**

W. C. McCormick and W. T. Kniker

In determining addiction to various analgesics two methods have been employed: (1) using lower animals in which tolerance and physical dependence are demonstrated, although this has proven unreliable except in monkeys, and (2) using humans. The use of human subjects falls into 2 groups. The first are those with intractable pain who require relief for long periods. In these a very low dose is given and a low grade dependence is produced. Due to the low dosage, signs of abstinence phenomena may be confused with signs of the disease. For this reason many of the new analgesics have been reported non-addicting by this method and later found to be addicting. The second group are prisoners who have been morphine addicts. This method has been reliable but time consuming and requires highly trained personnel; moreover, since these people at one time were addicts, re-addiction may be more easily obtained than in other people.

Aside from the emotional basis for drug addiction, the most satisfactory theory to explain addiction and the abstinence phenomenon is that of homeostatic changes. Certain homeostatic reactions, which oppose the action of the drug, are increased by repeated administration of the drug. When the drug is withdrawn these active mechanisms still continue to act and give rise to the abstinence symptoms. It has already been shown by Semura (1931), Sajo (1936), Saski (1938), and Kubo (1938) that tissues from the chick embryo develop a tolerance to lethal doses of morphine in tissue culture when grown in media containing increasing doses of the drug. Sajo also observed retardation of growth when these tissues were transferred to media without morphine, while Kubo and Saski state that actual injury of the cells occurs when they are transferred to drugless media. Okada (1935) showed that the leucocytes of chicks that

* From the Tissue Culture Laboratory, Medical Branch, The University of Texas, Galveston. Aided by a grant from Hoffman-La Roche, Inc., administered by C. M. Pomerat. Received for publication December 26, 1952. Grateful thanks are extended to George Crosby for help in this study.

had received repeated doses of morphine showed decreased motility in normal plasma and that leucocytes from chicks not having received the drug showed decreased motility when placed in plasma from the addicted chicks, indicating the development of tolerance on the part of the leucocytes to the effect of the morphine.

The present investigation was undertaken with the assumption that addiction is on a cellular basis and that by tissue culture methods a very rapid and inexpensive method of assaying drugs for the detection of addictive tendencies could be found not necessarily requiring human tissues. The first part of this effort was an attempt to duplicate the Japanese work, but with the use of the tissues of rats which had been addicted *in vivo*. The second part consists of a cytological analysis of the cell constituents after exposure to addicting doses of morphine. This type of investigation may very well be done with human material.

Methods. Twenty-six albino rats weighing from 100–150 grams were divided into 4 groups: 4 females and 2 males receiving daily injections of isotonic saline; 7 females receiving daily injections of morphine sulfate; 6 females receiving daily injections of Dromoran, and 4 females and 3 males receiving daily injections of sodium amytal. The dose of morphine sulfate was begun at 20 mg./kg. and increased 10 mg./kg. every 3 days, until a dosage of 240 mg./kg. was attained, the latter having been reported as an habituating regime by Stanton (1936). The Dromoran was administered in the same manner, but at concentrations one-third as great since it has been found to have three times the activity and toxicity of morphine sulfate by Pomerat (1950). Sodium amytal was also administered at the same dosage as morphine, but when a concentration of 190 mg./kg. was reached, the amount was decreased to three-fourths that of morphine sulfate because of the deaths of four rats in the amytal group.

Morphine sulfate and sodium amytal were administered as 1 per cent solutions for the first 9 days, then as 2 per cent solutions for the next 30 days, and thereafter as 3 per cent solutions. The Dromoran was administered at one-third this concentration. The rats were weighed every third day and the amount of drug increased at that time. All injections were given subcutaneously in the skin of the back or abdomen.

After a dose of morphine sulfate of 240 mg./kg. was reached, one rat from each series was killed and tissues set up *in vitro*. Three weeks later another animal from each series was killed and the tissues grown in the same way. At this time it was decided that the dose of morphine should be increased above 240 mg./kg., so that in the next 3 weeks the doses were gradually raised to 350 mg./kg. with a comparable increase in Dromoran,

at which time the third series of animals was killed and their tissues were explanted. When this experiment was analyzed it was discovered that the tissues still did not need the drug for growth in culture; therefore the dose of morphine was increased to 500 mg. with a comparable increase in Dromoran over the next four weeks, and the fourth series of animals killed and their tissues explanted.

For the final experiment, one rat addicted to morphine and one control animal were given 630 mg./kg. of morphine and one rat addicted to Dromoran and control animal were given 210 mg./kg. of Dromoran. Within 6 hours all rats but the one addicted to Dromoran were dead. The tissues were again explanted but without adding any drug to the nutrient fluid.

Observations of the Rats. After the first week of injections, all the rats receiving Dromoran and morphine sulfate began to exhibit an intense inflammatory reaction with consequent scarring and loss of hair at the site of every injection. The drugs were then neutralized with an excess of $NaHCO_3$ just before injection and the inflammatory reaction was decreased.

In the course of the habituations the animals receiving Dromoran and morphine sulfate gained weight at approximately the same rate, which was about equal to the controls for the first 3 weeks; then there was a more gradual gain in weight with periods of no gain which continued for the remainder of the experiment. The sodium amytal and control rats showed a steady rise in weight until the time of death, that of the sodium amytal animals being only a little less than that of the controls. The average weight for the morphine sulfate females increased from 130 gm. to 205 gm. by the end of the habituation; the average weight of the Dromoran rats changed from 120 gm. to 200 gm.; the remaining female receiving sodium amytal increased from 120 gm. to 240 gm.; and the one remaining female control increased from 130 gm. to 250 gm.

Males under sodium amytal averaged an increase from 130 gm. to 325 gm., whereas the remaining male control gained from 135 gm. to 335 gm.

The rats exhibited no behavioral patterns suggestive of "desire" to receive their daily injections. Those animals receiving morphine sulfate and Dromoran appeared weak and listless but were hyperirritable whenever handled. Rats receiving sodium amytal behaved like those of the control series.

After approximately 3 weeks of injections, the rats receiving sodium amytal were regularly found to be completely anesthe-

tized a few minutes after each injection for an increasing duration, varying directly with the increases in sodium amytal concentration. After injections of morphine sulfate, rats exhibited rigidity, were stuporous, and assumed abnormal positions, such as lying on their backs, or leaning against the sides of the cage while standing on their haunches and often gnawing on the cages. Rats, after receiving Dromoran, clutched pellets of food in their mouths, assumed rigid upright positions and were hyperexcitable.

Unfortunately, 4 controls died after receiving injections of sodium amytal rather than their usual isotonic saline; 4 rats were lost during their "habituation" to sodium amytal. More controls were obtained which were of the same age as the original controls and the experiment continued.

Tissue Culture Methods. For the first series of rats the following concentrations of drugs were incorporated in the nutrient medium:

	Morphine Rat	Dromoran Rat	Sodium Amytal Rat
	(Concentrations in gamma/ml.)		
1	120	40	90
2	240	80	180
3	1200	400	900
4	2400	800	1900
5	6000	2000	4500
6	Control	Control	Control

Tissues of the control rat were grown at all of the above concentrations.

The following tissues were taken from each rat. The numbers show the quantity of original explants for each concentration used.

Corpus callosum	6
Central gray nucleus	6
Skin	12
Omentum	12

The tissues were set-up in roller tubes with 3 explants on a cover slip, measuring 12 x 50 mm. The clot was made by mixing the drug at 4 times final concentration with an equal quantity of embryonic extract. This mixture was then combined with an equal amount of plasma containing 4,000 units of penicillin/ml. and rabbit serum to make the clot, that is:

Drug (4x final conc.)	25%	50%	100%
Embryonic extract	25%		
Plasma + penicillin	25%	50%	
Rabbit serum	25%		

After the clot was formed 2 ml. of fluid nutrient was added to each tube. The fluid phase consisted of an equal mixture of Gey's balanced salt solution, containing 2 times the final concentration of the drugs and human ascitic fluid (cirrhosis of the liver) with 5 per cent embryonic extract, or:

Gey's solution + 2x final conc. of drug...	50%	100%
Ascitic fluid	45%	
Embryonic extract	5%	

To this fluid 1,000 units of penicillin/ml. were added. The tubes were then placed in drums in an incubator where they rotated 8 times per hour at a temperature of 37° C.

The fluid nutrient was changed every third day and the tissues were checked for the amount of outgrowth every second day. At the end of 10 days 6 explants from each tissue for each concentration were fixed in methyl alcohol and stained with Jacobson's method.

The second series was set up in the same manner, but with the following drug concentrations:

	Morphine Rat	Dromoran Rat	Sodium Amytal Rat
	(Concentrations in gamma/ml.)		
1	120	40	90
2	240	80	180
3	480	160	500
4	720	240	1000
5	960	320	2000
6	Control	Control	Control

The control rat tissues again received each of the above concentrations of each drug. Tissues used and number of explants per concentration were:

Corpus callosum	6
Lung	6
Skin	12
Omentum	12
Testes (amytal only)	6

All explants were fixed and stained with Jacobson's method at the end of 10 days.

In the third series the method was the same but the following concentrations were used:

	Morphine Rat	Dromoran Rat	Sodium Amytal Rat
	(Concentrations in gamma/ml.)		
1	120	40	No animals
2	240	80	remaining
3	480	160	
4	720	240	
5	1440	480	
6	Control	Control	

The control rat tissues received each of the above concentrations of both drugs. The following tissues, with number of explants for each concentration, were used:

Cortex	9	Omentum	9
Lung	9	Liver	9
Skin	9		

Three explants for each concentration were fixed and stained with Jacobson's on the 2nd day, 3 more on the 6th day, and 3 more on the 10th day of the experiment.

In the fourth series the same drug concentration and tissues were utilized. All cultures were fixed with methyl alcohol and stained with Jacobson's stain on the 10th day of the experiment.

In the final experiment the following tissues with number of explants were used:

Cerebellum	15	Omentum	15
Cortex	15	Liver	15
Skin	15		

No drugs were used in this experiment. All tissues were fixed and stained on the 10th day of tissue culture.

Observations on the Cultures. In the first series all but the first 2 concentrations of the drugs were too high and no growth occurred. There was no appreciable difference in growth of the addicted and controlled animals except for a more rapid outgrowth of the omentum in the addicted animals. This had occurred by the end of 48 hours in the addicted animals, and at the end of 72 hours in the control animal.

In the second series, cells of the addicted animals showed a tolerance to lethal doses of morphine sulfate and Dromoran (see Fig. I), but injury of the tissues was not noted when grown in medium without morphine. Outgrowth in the control solution was equal to or slightly greater than the outgrowth in the first concentration of morphine (120 gamma/ml.) which was the drug level allowing for greatest amount of outgrowth. Various bizarre and multinucleated cells were present in the epithelial elements in the tissues receiving the drug, but this occurred to the same extent in the control as well as the addicted animals. The lethal dose of sodium amytal was the same for addicted and control animals; therefore, by this method the cells could not be shown to have developed a tolerance to a lethal dose of sodium amytal.

In the third series the same results were again obtained. In this series outgrowth did not occur at as high a concentration as in the previous one, although the doses of the drug to the animals was higher, but again tolerance to a lethal dose of Dromoran or morphine was demonstrated. The liver of the addicted animals did not grow. The liver of the control animals grew but its outgrowth was only fair.

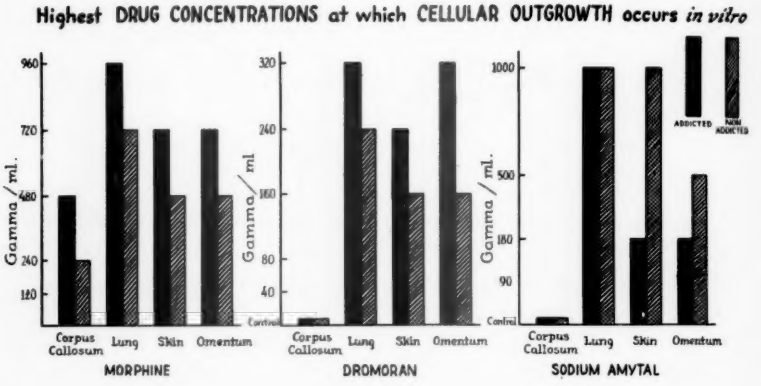


FIGURE I

The results of the fourth series were like the third. Outgrowth was poorer than in the second experiment, but this was uniform for the addicted as well as control animals.

In the final experiment, the cerebellum and lung from all the animals grew very well. These tissues from the addicted animals showed no injury when grown in media without the drug. The skin and cerebral cortex from all animals grew poorly. The liver from the control animals grew, but that from the addicted animals did not grow.

Discussion. It was the purpose of these experiments to discover whether the tissues of rats addicted to morphine, Dromoran, and sodium amytal would exhibit a physical dependence for the drug in tissue culture. This we failed to demonstrate, but such tissues did show a tolerance to a lethal dose of the drug except in the case of sodium amytal. This cellular tolerance must represent homeostatic changes occurring within the cells

themselves. Thompson and Gross (1941) reported that dogs tolerant to morphine were able to destroy or inactivate morphine to a much greater extent than normal animals. In tolerant animals 16 per cent of the morphine was found to be in a form easily hydrolyzable and 29 per cent in a form difficult to hydrolyze, whereas in non-tolerant animals only 8 per cent was easily hydrolyzable and 66 per cent was difficult to hydrolyze. If these changes in morphine are due to generalized tissue reaction rather than to one single organ such as the liver, it may well explain the tolerance to a lethal dose of the morphine and Dromoran.

Kubo (1938) and Saski (1938) noted actual injury and death of cells when "addicted" tissues were placed in drugless media. These authors started with chick tissues *in vitro*, "addicted" them to the morphine and then withdrew the morphine, whereas in the present experiments the rats were "addicted" *in vivo* and then the tissue studied *in vitro* by the tissue culture method. For this reason the two experiments are not exactly comparable.

Another interesting observation was that the liver of the "addicted" animals did not grow in tissue culture. Possible explanations for this are: (1) the liver was dependent upon the morphine or Dromoran for growth; (2) the liver of the rats was injured by the drug to the extent that growth did not occur; (3) the concentration of drug in the liver was so high that outgrowth did not occur. The first explanation may be ruled out since growth did not occur if morphine or Dromoran were added to the tissues. Unfortunately no liver tissue was saved for histological study or assay; however, the last explanation seems most feasible in view of the fact that morphine is known to be largely excreted by the liver. In regard to sodium amytal neither the rats nor the tissues in culture exhibited a tolerance to a lethal dose of the drug.

Conclusion

The tissues of rats addicted to Dromoran and morphine sulfate showed a tolerance to a lethal dose of the respective drug when grown in tissue culture. No tolerance was developed in those animals receiving sodium amytal. No dependence on the drug was demonstrated by the tissues in tissue culture.

REFERENCES

Kubo, Taizo: Studien über die Morphinentwohnung beim Kulturegewebe. *Jap. J. M. Sc. IV, Pharmacology*, 11: 142–143 and 143–144, 1938.

Okada, Sozaluro: Experimentelle Studien über die Morphingewohnung an den Kulturen von Hubenenleve Koyten. *Jap. S. M. Sc. IV, Pharmacology*, 8: 44, 1935.

Pomerat, C. M.: Personal Communication, 1950.

Sanjo, Kano: Experimentelle unter Suchungen über die Gewohnung der Iris Epithelkulturen an Morphin. *Jap. J. M. Sc. IV, Pharmacology*, 9: 13, 1936.

Saski, Morio: Studies on the Phenomenon of Abstinence of the Morphinized Culture *in Vitro*, and the Curative Effect of Morphine and Its Derivations on It. *Jap. J. M. Sc. IV, Pharmacology*, 9: 29, 1938.

Semura, Seucki: Ueber den Einfluss Verschiedner Pharmaka der Morphingruppe aus das Wachstem der *in Vitro*. Kulturen Von Fibrolastem, nebst linem Studiurn über die Morphingewohnung der Gezuchtesten Gemebe. *Jap. J. M. Sc. IV, Pharmacology*, 5: 54, 1931.

Stanton, Eugene J.: Dihydromorphine Hydrochloride. *J. Pharmacol. Exper. Therap.*, 56: 252–263, 1936.

Thompson, V., and E. G. Gross: Excretion of Combined Morphine in the Tolerant and the Non-tolerant Dog. *J. Pharmacol. Exper. Therap.*, 72: 138–146, 1941.

THE USE OF YOLK SAC CULTIVATED BACTERIA IN BACTERIAL STUDIES

GEORGE F. MCKENNA AND ALFRED TAYLOR*

In chemotherapy work there is always a need for a simple and relatively quick method of testing extracts as antibacterial agents *in vivo*. Yolk sac cultures seemed to provide the most practical medium for these experiments. The use of yolk sac cultured tumors (1) in cancer chemotherapy has been successful, as observed by Taylor and Carmichael (1948), and affords a very easy and quick method for testing as reported by Taylor, Thacker, and Pennington (1942) and Taylor *et al.* (1943 and 1948). Therefore, it was decided to try and work with yolk sac cultivated bacteria.

Materials and Methods. The nine species of bacteria listed below have been used successfully for antibacterial tests in the chick embyro.

1. *Staphylococcus aureus* FDA 209.
2. *Escherichia coli* ATCC 9637.
3. *Proteus vulgaris* ATCC 9623.
4. *Bacillus subtilis* ATCC 6633.
5. *Salmonella paratyphi* A ATCC 9444.
6. *Salmonella paratyphi* B ATCC 10258.
7. *Salmonella typhosa* ATCC 6229.
8. *Pseudomonas aeruginosa* ATCC 10197.
9. *Shigella dysenteriae* ATCC 9665.

After using various dilutions of a test organism, it was found that a 1:1,000,000 dilution of a 20 hour culture in .85 per cent saline is quite satisfactory for all species except *Bacillus subtilis*. This species is used in a dilution of 1:100,000. The dilutions are prepared by recognized sterile bacteriological techniques.

Preparation of Eggs. The eggs are candled after four days of incubation. These eggs can be used, or one can wait until the eggs have incubated 9 or 10 days. Regardless of the age, the eggs are prepared for inoculation in the same manner. On the morn-

* From the Biochemical Institute and Pharmaceutical Foundation, The University of Texas and the Clayton Foundation for Research, Austin.
Received for publication January 26, 1953.

ing the eggs are to be used, they are candled and an area about ¼″ in diameter is circled in the lower half of the air sac. At the same time a small circle about ¼″ in diameter is marked over the blood vessels of the chlorio-allantoic membrane well below the embryo amniotic sac and large blood vessels. The shell area within these circles is scrubbed with 70 per cent alcohol, then dented with a moderately blunt scissor point, being especially careful that the shell membrane is not broken. The scissor point is flamed for sterilization before each denting operation.

After each egg has received the two dents, it should be carefully handled so that the position of blood vessels and dent over them are not appreciably altered. If desired, the egg can be candled after the first injection into the yolk sac, and the area over the blood vessels marked, scrubbed, and dented. After some experience in handling the eggs, the two dents can be made at the same time.

Injection of Bacteria. Bacteria are injected by means of a 1cc tuberculin syringe and a 22 gauge needle, 1¼″ in length. The most satisfactory quantity is 0.1cc of 1:1,000,000 dilution. All bacteria are injected through the dent over the air sac into the yolk sac, inserting the needle its full length for the injection. All eggs are returned to the incubator (37.2° C.) for one hour before the second injection. This injection is made through the dent over the blood vessels in the manner shown and described by Taylor (1950).

This injection is made by inserting the unknown solution, commercial antibiotic, and sterile .85 per cent saline control or sterile distilled water controls. Commercial antibiotics are used for comparison, and 0.1 mg. per egg is a very satisfactory dosage. Penicillin, aureomycin, streptomycin, and chloromycetin have been used.

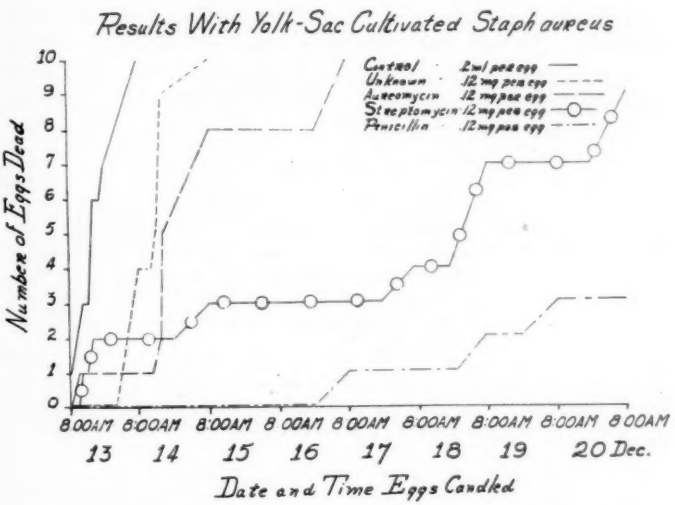
Results

The commercial antibiotics prolong the life of the chick embryo considerably. In many cases the eggs are opened and the embryos weighed. The results in figure I show how much the life of chick embryos is extended by commercial antibiotics. Also the unknown is shown to have some effect, but not nearly so much as the commercial antibiotics. Ten eggs are used for

each of the substances shown. This can be repeated and has been many times.

Discussion. Several disadvantages of the method have been noted. Only the percentage survival over a period of hours or days is secured. One can only compare survival in these types of tests. Some might object to chick embryos because the method of breathing is entirely different from mice, rats, or dogs. Since this method is for preliminary testing or screening, additional tests will have to be made with other laboratory animals.

FIGURE I



The eggs are not opened but candled to determine whether the chick is still alive. The effect on the various organs is not known. Again, additional work will have to be done with unknowns that show promise.

The main advantage of the chick embryo method for antibacterial tests is that it is relatively inexpensive in comparison to other *in vivo* tests. The problem of cages and feed are not encountered when working with eggs. Usually a small oven or incubator is sufficient. Sterile conditions are more easily maintained in egg work than in work with other experimental

animals. A large number of extracts can be tested in a few days or less. Plate tests are used in conjunction with this, and only those showing any promising results are tested in eggs. In this manner we have simplified our screening to one *in vitro* and one *in vivo* test, and both are relatively simple.

A large number of extracts have been tested in this manner. It was proven quite satisfactory as a technique for screening unknown solutions that gave positive results *in vitro*.

Conclusion. A method of injecting bacteria into the egg yolk sac and unknown substances over the blood vessels for evaluation as an antibacterial agent has been presented. The method is used for screening operations and gives results that compare rates of survival over a period of hours or days. Several bacteria have been used with varying degrees of success. Commercial antibacterial agents are used with very good results and comparisons can be made.

REFERENCES

Taylor, A., J. Thacker, and D. Pennington: Growth of Cancer Tissue in the Yolk Sac of the Chick Embryo. *Science*, 96: 342–343, 1942.

Taylor, A., R. E. Hungate, and D. R. Taylor: Yolk Sac Cultivation of Tumors. *Cancer Research*, 3: 537–541, 1943.

Taylor, A., and N. Carmichael: Yolk Sac Cultivated Tumor Tissue and Experiments in Tumor Chemotherapy. I. Riboflavin. *Texas Rep. on Biol. and Med.*, 6: 504–512, 1948.

Taylor, A., N. Carmichael, and T. Norris: A Further Report on Yolk Sac Cultivation of Tumor Tissue. *Cancer Research*, 8: 264–269, 1948.

Taylor, A: The Use of Yolk Sac Cultivated Tumors in Cancer Chemotherapy Studies. *Texas Rep. on Biol. and Med.*, 8(2): 227–237, 1950.

ISONIAZID IN THE TREATMENT OF TUBERCULOUS MENINGITIS*

JOHN W. MIDDLETON AND A. N. LONGFIELD

A year ago the problem of tuberculous meningitis was reviewed with particular reference to recent results in treatment of adults at the Medical Branch Hospitals. Shortly after that isoniazid (isonicotinic acid hydrazide) was introduced, and has been used in the treatment of each of the three adult cases of tuberculous meningitis reported here. These comprise consecutive cases and it is considered noteworthy that all have made at least a temporary recovery, whereas only one of the four cases previously reported survived even a few months.

Case 1. M. H., J. S. H., No. 70191, a white male, 35 years old, was admitted to John Sealy Hospital, Tuberculosis Ward, on April 17, 1952, with complaints of severe frontal headaches, anorexia, insomnia, and progressive mental confusion for the preceding four weeks. His referring physician had made a diagnosis of tuberculous meningitis on chemical and cellular changes in the spinal fluid in addition to acid fast bacilli on smear, and the patient had been treated with dihydrostreptomycin 1 gram twice daily, and sodium paraamino salicylate 14 grams daily for 3 weeks before admission. In spite of this, his condition had deteriorated. He had a past history of pulmonary tuberculosis and tuberculous tonsillitis two years previously, a year later surgical treatment of tuberculous orchitis, and four months before admission had a resection for tuberculous enterocolitis.

The patient showed disorientation as to time and place, but not to person, as well as confusion on admission. He had a left internal strabismus. There was no definite neck stiffness. Examination was otherwise normal. Spinal fluid examination initially showed 139 cells per cubic mm. with 60 per cent neutrophiles, 40 per cent leukocytes, 377 mg. chloride and 15 mg. of glucose.

An electroencephalogram was obtained because of petit mal seizures and was diffusely abnormal, compatible with a space occupying lesion of the frontal area probably on the left. On chest X ray there was scarring of the left upper lobe. Skull films were normal. Other laboratory studies did not reflect abnormality.

The patient was given 200 mgs. of isoniazid 4 times daily for 2 weeks without change in his physical condition or mental status, but with slight improvement in spinal fluid findings. He was then started on dihydro-

* From the Department of Internal Medicine, The University of Texas Medical Branch, Galveston.

streptomycin sulfate 1 gm. intramuscularly every 12 hours in addition. On the combined schedule there was gradual but progressive clearing of his mental status, neurological findings, and electroencephalogram. His spinal fluid findings on May 6, 1952, six weeks after starting isoniazid and 4 weeks after resumption of dihydrostreptomycin showed cells 34 per cubic mm. with 12 per cent neutrophiles and 88 per cent lymphocytes, chlorides 440 mg., glucose 50 mg., and protein 75 mg. per 100 cc.'s. Smears and cultures of the weekly spinal fluid specimens were negative bacteriologically. The patient was discharged to home care by his family physician on June 15, 1952, with plans to continue his treatment unchanged for a minimum of 18 months.

Reports received from the family physician through January 25, 1953, indicate that the patient has continued to improve symptomatically. The patient has complained of some deafness in the left ear but audiometric tests show only very slight loss of high tone hearing bilaterally. Spinal fluid examinations have been normal for six months. A progress electroencephalogram on November 5, 1952, was compared with previous tracings made here and interpreted as a normal tracing with complete clearing of the focal disturbance previously seen.

Case 2. A. N., J. S. H. No. 11247-I, a 23-year-old white female was admitted July 12, 1952. In June, 1951, she had developed ascites and had been treated elsewhere with paracentesis of 4,000 cc.'s of clear amber fluid. It was known at this time that her husband had far advanced pulmonary tuberculosis, and an X ray of her chest was interpreted as showing minimal tuberculosis. She continued to have intermittent abdominal pain with right upper quadrant tenderness and in May, 1952, another X ray showed a left pleural effusion. In July, 1952, she began to have headache and increased amounts of abdominal pain with nausea and vomiting. She was admitted to another hospital where she was noted to have a moderately stiff neck. Lumbar puncture showed cell count of 440 cells with 71 per cent neutrophiles, 29 per cent lymphocytes. Spinal fluid sugar was 48 mg. per 100 cc.'s, protein 127 mg. per 100 cc.'s and chloride 660 mg. per 100 cc.'s as sodium chloride. Spinal fluid examination two days later was essentially the same except for a higher elevation of the protein to 168 mg. per 100 cc.'s. She was transferred to John Sealy Hospital and admitted to the Tuberculosis Ward on that day. On admission the patient showed emaciation and dehydration with a drawn expression. There was severe stiffness of the neck but no opisthotonos. The chest showed dullness and diminished breath sounds over the left chest laterally and posteriorly. The abdomen showed generalized tenderness with a fluid wave in both flanks. The patient was nauseated. Initial spinal fluid here had a ground-glass appearance. It was obtained under no increase in pressure. A pellicle formed immediately. There were 8 per cent polymorphonuclear leukocytes and 92 per cent lymphocytes, with 128 mg. per cent of protein. Sugar was 41 and chlorides as chloride ion 416. The patient received fluids by the intravenous route for the first ten days of hospitalization and dur-

ing that time received 1 gm. of dihydrostreptomycin sulfate intramuscularly every 12 hours. On July 23, she became able to tolerate fluids and food by mouth and she was started on isoniazid 100 mg., 4 times daily. Her initial weight was 90½ pounds or 41 kilos so that this represented a dosage of 8 mg. per kilo of body weight per day. There was progressive improvement in the patient's physical status and on the 47th hospital day she was started on sodium PAS 3.5 gm., 4 times daily. At this time the patient was essentially asymptomatic, having recovered from her nausea, vomiting, headache, and neck stiffness, and with resorption of her ascites without paracentesis. Spinal fluid examination done at weekly intervals showed progressive, steady decrease in the number of cells and in the protein, increase in the spinal fluid sugar and chloride. The last spinal fluid examination on November 17, 1952, showed crystal clear fluid with 52 cells per cubic mm., with a differential of 10 per cent polymorphonuclear leukocytes and 90 per cent lymphocytes; 89 mg. of protein per 100 cc.'s, 54 mg. of sugar per 100 cc.'s with 418 mg. of chloride as chloride ion. Smears and cultures for acid fast bacilli, pyogenic organisms and fungi were repeatedly negative. The patient was discharged on December 9, 1952, five months after admission, asymptomatic, much improved. It is planned to continue treatment for a probable total of 18 months.

Case 3. D. C. T., J. S. H. No. 15, a 39-year-old white female was admitted to John Sealy Hospital on September 5, 1952. She became ill 3½ weeks earlier, with dyspnea, edema, diarrhea, bloody stools, nausea, and vomiting. She then developed severe frontal headache. Her referring physician obtained an X ray of her chest, which was interpreted as showing miliary tuberculosis. She had become somnolent 4 days before admission. One sister had died at this hospital of far advanced pulmonary tuberculosis 4 years previously.

Examination on admission revealed an extremely ill, feverish, emaciated, dehydrated woman. Temperature was 103 degrees F. rectally, pulse 120, and respiration 40 per minute. The left pupil was dilated, both pupils reacted to light. Examination of the chest was essentially negative. There was questionable neck rigidity.

On admission spinal fluid was obtained under normal pressure. It was cloudy and a pellicle formed. There were 333 cells per cubic mm. with 21 per cent neutrophiles, 79 per cent lymphocytes, chlorides 346 mg., sugar 18 mg. and protein 110 mg. per 100 cc.'s. The fluid was bacteriologically negative and remained so. X-ray of the chest showed a fine uniformly distributed discreetly granular infiltration throughout both lung fields compatible with miliary tuberculosis, with infiltration and cavitation of the right apex. Electrocardiogram showed nodal rhythm. The remainder of laboratory studies were not remarkable.

The patient was hydrated with intravenous and intragastric feedings and given isoniazid 100 mg. t.i.d. and 150 mg. at bedtime on an estimated dosage schedule of 10 mg. per kilogram of body weight. Dihydrostreptomycin sulfate 1.0 gm. was given intramuscularly every 12 hours. Oxygen

by tent was required for 3 weeks. By this time the patient had improved sufficiently to take oral feedings and sodium PAS 3.0 gm., 4 times daily was started. Her mental and physical condition continued to improve. X ray of the chest showed decrease in areas of cavitation of the right apex by November 17, 1952, with no change in the miliary infiltrate. The weekly spinal fluid examination showed a consistent decrease in cells, increase in sugar and chlorides without much change in protein, so that on November 17, 1952, fluid was clear, had 17 cells per cubic mm. with 9 per cent neutrophiles, 91 per cent lymphocytes, chlorides 402 mg., sugar 51 mg., and protein 112 mg. per 100 cc.'s. The patient's family elected to continue treatment at home 10 weeks after admission. The patient was conscious, oriented, and much improved. It is planned to continue therapy with dihydrostreptomycin, sodium PAS, and isoniazid for a minimum of 18 months.

It is to be noted that no attempt was made after the initial case to treat with isoniazid alone. Clark (1952) and his co-workers have reported on the use of isoniazed in military and meningeal tuberculosis with similar results in patients who received both streptomycin and isoniazid.

Because of the gravity of these cases a rather large dose of isoniazid was used without untoward side effects. None of the cases received streptomycin intrathecally, although the authors were previously convinced of its desirability.

No attempt has been made to draw any conclusions from such a short series of cases. The necessity of further studies and longer periods of observation is obvious.

Summary. Recovery of three consecutive cases of tuberculous meningitis in adults, treated with streptomycin and isoniazid is reported.

REFERENCES

Clark, C. M., D. F. Elmendorf. Jr., W. U. Cawthon, C. Muscheim, and W. McDermott: Isoniazid (Isonicotinic Acid Hydrazide) in the Treatment of Miliary and Meningeal Tuberculosis. *Am. Rev. Tuber.*, 66: 391, October, 1952.

Middleton, J. W., and A. N. Longfield: Tuberculous Meningitis. *Texas Rep. on Biol. and Med.*, 10:629–638, 1952.

THE SENSORY INFLUENCES OF THE SKIN

M. F. Ashley Montagu*

The title of this paper implies no artificial dichotomy. The organism is a whole, and it is implied that the total organism is essentially a nervous system which may be organized to function as a whole. Biological organization and psychical activity may not be quite the same things, but fundamentally they are almost certainly different aspects of the functioning of the same thing. It must be recognized, however, that people will continue to talk of body *and* mind, as if they were really separable quantities. If the terms body and mind are to be retained, mind should be understood as an aspect of the functioning of the body, as the behavior of the organism. Mind is body acting under the influence of and in relation to environmental factors which always affect the behavior of the organism.

In this discussion we shall be concerned with the skin *acting;* the skin as initiator and as at least a necessary condition of behavior. A somewhat different approach to the skin than is customary is proposed: a centripetal as contrasted with the reigning centrifugal approach to the skin of contemporary psychosomatic medicine. The very important mechanisms by means of which changes in the skin are produced by what goes on in the cerebral cortex or the diencephalon will not be considered, but rather attention will be given to the skin as a system affecting the cerebral cortex, the thalamus, and the hypothalamus. Because what is so obvious or should be, is for that reason so often overlooked, it may be desirable here to recall the fact that the skin and the nervous system are derived from the same blastocystic ectodermal layer.

What influence has what the skin undergoes or doesn't undergo upon the development of the organism? Will it be an exaggeration to say that so far as most students of the human organism are concerned the skin has been of little or no relevance? No one seems to have asked the question: What does such a large representation of the tactile-cutaneous sense in the brain

* From the Department of Anthropology, Rutgers University, New Brunswick, New Jersey. Presented at a special seminar held at The University of Texas Medical Branch, Galveston, April 1, 1952. As presented this paper was originally entitled "The Mind of the Skin."

signify? May there not be a deeper significance to this extensive representation than is customarily indicated?

In 1944 a study was begun on the nature of the basic needs of the developing human being from a scientifically eclectic and integrative point of view. As one considers the various forms of behavior which answer to the requirements of a basic need, cutaneous stimulation looms more and more frequently upon the horizon. Some extremely interesting facts keep coming up again and again—facts known to everyone.

One of the best ways of determining whether a particular function is basic or not, is to discover how widely distributed it is in the class of animals to which the propositus belongs, for what is phylogenetically basic is likely to be physiologically significant. So let us go to the lower animals. We may be able to learn something from them about the human skin.

Let us begin at birth. What do we observe among mammals generally, at birth? One of the first things of relevance which we perceive is that the mothers spend a great deal of time licking their young, particularly in the perineal region. We have usually dismissed this observation with the remark that the mother is "washing" her young. Mothers wash their young, there is nothing remarkable about that. But is it merely "washing"? During the process of being licked the young obviously receive a great deal of cutaneous stimulation. Why? Does this cutaneous stimulation stop with the skin or does it have any further functions?

Obviously one of the ways in which to find the answer to this question is to discover what happens to the young when they are not licked. This can be determined by experimental means. Fortunately, there are already available a number of unpremeditated experiments of this sort, which are of all the more value since they were not set up in order to test any hypothesis in relation to the skin. Meanwhile it will be of interest to see what inquiry reveals among those who should know, namely, animal breeders, husbandrymen, verterinarians, and the staffs of zoological gardens.

When one asks persons of such experience, those who have any positive observations to offer reveal a significant unanimity in the kind of effects they report. The substance of this is that if the newborn animal is for some reason not licked, particularly

in the perineal region, it is likely to die of a failure to function of the gastro-intestinal system or of the genito-urinary system.

Here, then, we have a hint concerning the possible function of cutaneous stimulation. The indication is that it serves to activate or assists in the activation of the gastro-enteric and genito-urinary tracts. Before citing some of the evidence let us ask how, by what means, cutaneous stimulation may activate the gastro-enteric and genito-urinary tracts. It is probable that the stimulation of the peripheral sensory nerves of the skin is a necessary part of the process of activating these essential systems.

It is suggested that stimulation of the peripheral sensory nerves of the skin is a necessary part of the process of activating the gastro-intestinal tract through the connection of the peripheral and central nervous systems with the autonomic. That, in many cases at least, when cutaneous stimulation is inadequate the autonomic is inadequately stimulated and there is a failure of activation of the gastro-intestinal tract. It is very likely that one or more other sustaining systems of the body are similarly involved. So far as the functioning of the genito-urinary system is concerned the evidence derived from the study of rats and dogs indicates that this will simply not function in the absence of cutaneous stimulation in the region of the perineum. The most interesting evidence on this point comes from Professor James A. Reyniers of the Laboratories of Bacteriology of the University of Notre Dame. At the Lobund Laboratories at Notre Dame Professor Reyniers and his group have been interested in raising germ-free animals. In the early days of their experiments they found that their animals died apparently from a failure of the genito-urinary and gastro-enteric systems. Miss deBrusa of the San Diego Zoological gardens brought her own observations on the care of the newborn by their mothers to bear upon the solution of this problem, advising the Notre Dame group of researchers to stroke the perineum of the young animals after each feeding until urination or defecation occurs.

In a personal communication, Professor Reyniers writes: "With respect to the constipation problem in hand-reared newborn mammals the following may be of some interest: Rats, mice, rabbits and those mammals depending upon the mother

for sustenance in the early days of life apparently have to be taught to defecate and urinate. In the early period of this work, we did not know this and consequently lost our animals. The unstimulated hand-fed young die with an occlusion of the ureter and a distended bladder. Although we had for years seen mothers licking their young about the genitals I thought that this was a matter largely of cleanliness. On closer observation, however, it appeared that during such stimulation, the young defecated and urinated. Consequently, about twelve years ago, we started to stroke the genitals of the young after each hourly feeding with a wisp of cotton and were able to elicit elimination. From this point on we have had no trouble with this problem."

These observations confirm those which are known to many animal breeders, dog fanciers, veterinarians, and staff members of zoological gardens, but references to such observations do not appear in the literature.

According to Windle the human fetus is capable of urination and defecation *in utero*, and apparently its external genitalia require no cutaneous stimulation in order to function, but whether this is also the case in all other members of the Order Primates to which man belongs we do not know.

Thirty years ago Hammett quite inadvertently found in the course of a series of experiments of an operative nature that "gentled" rats, that is, rats which had been stroked, were far better operative risks than those which had not been so "gentled." In the ungentled rats he found that the picture as a whole is one of constant high irritability and neuromuscular tension in contrast with the rather friendly behavior of the "gentled" rats. In the "ungentled" series 79 per cent died in parathyroid tetany within 48 hours of thyroparathyroidectomy, whereas only 13 per cent of the "gentled" rats died of the same cause within the same period. The same ratios were maintained when the rats were parathyroidectomized alone. "Ungentled" rats when "gentled" had their mortality rate reduced to zero in the relatively small series studied.

Hammett's observations indicate something of the possible importance of "gentling" or cutaneous stimulation for the viability of the organism under conditions of stress. We may recall

here Osler's aphorism: "Taking a lady's hand gives her confidence in her physician." We may also recall the fact that taking almost anyone's hand under conditions of stress is likely to give both the taken and the taker a feeling of greater security by reducing anxiety. Here we must proceed to ask the question: What evidence is there for the suggested relationship of any other sustaining system with cutaneous stimulation? The respiratory system would seem to be an obvious one.

For several thousand years, at least, it has been known that if the newborn child doesn't breathe a hearty slap or two on the buttocks will often be sufficient to induce breathing. This is a very remarkable fact the physiological significance of which does not appear to have elicited much interest. On the basis of the physiological relations already briefly indicated it seems that under similar conditions stimulation of the respiratory centers and apparatus could be achieved by subjecting the non-breathing baby to immersion in hot and cold baths. Upon inquiry it was found that this is, indeed, an age-old procedure. In such cases it would appear to be a reasonable assumption that it is the cutaneous stimulation which activates the autonomic which in turn acts upon the respiratory centers and viscera. The effect of a sudden cold shower upon the respiratory system is too well known to require stressing here.

May it not be that the contractions of the uterus during labor represent, in addition to their other functions, a series of massive cutaneous stimuli calculated to activate such vital systems as the respiratory, and gastrointestinal? This is a question which, to some extent, can be answered. Caesarian-delivered and some prematurely-born infants ought to throw some light on this. If there have been no contractions of the uterus or delivery has been precipitate the amount of uterine cutaneous stimulation which the infant will have undergone will be minimal. Upon the hypotheses, then, which have been postulated in this paper such infants should exhibit a higher frequency of respiratory and gastrointestinal difficulties. Unfortunately, the data for caesarian and prematurely-born infants at birth is not available on these points, but evidence is available for prematures during the first year of life, and also as older children.

Drillien found that prematurely-born children showed a significantly higher incidence of nasopharyngeal and respiratory troubles than normally born children, and that this difference was especially marked in the first year. Shirley found that, among other things, prematurely-born children achieve bowel and bladder sphincter control later and with greater difficulty than do children born at term. Both Shirley and Drillien observe that prematures as children presented more frequent and greater feeding problems than children born at term. These observations suggest the possibility of inadequate cutaneous stimulation playing a role here and, in some cases at least, resulting in a greater susceptibility to infection and disorder of the respiratory and gastrointestinal systems.

Love has been defined as the harmony of two souls and the contact of two epidermes. A truth spoken in jest, it hits off at least one fact which is too often overlooked, the contact of epidermes. Cutaneous contact between infant and mother is obviously of some organismic value to the infant. It is obvious to anyone who has ever observed an infant snuggling up to its mother's body that it derives pleasurable sensations from that contact. Cutaneous contact between infant and mother usually constitutes the first act of communication between them, and cutaneous contact would seem to be the language which for some time the infant best understands. It may, perhaps, be recalled here not altogether without relevance that it was through the skin that both Helen Keller and Laura Bridgman were first reached and through which they learned to communicate with the rest of the world.

The relationship of the baby's lips to the mother's nipple and breast is undoubtedly one of the most pleasurable of such early experiences, and it is desirable to note that much of the cutaneous stimulation which the individual subsequently experiences is largely associated with stimulation of the lips. The lips are, of course, eversions of the oral mucous membranes and are not strictly speaking cutaneous at all—the relationship is, however, very close. As is well known the representation of the lips in the cerebral cortex is simply enormous as compared with other parts of the body. It is of interest to note that in fear, shock, worry, or weeping, the hand is frequently applied

to the lips, as if to secure succor and support, and that under conditions of stress persons often go into labored breathing reminiscent of breathing at birth. Stroking, caressing, of the skin under such conditions will often serve to restore the sufferer to equilibrium. It is known that in some cases an attack of asthma may be instantly relieved by putting a sympathetic arm around the sufferer.

It is fairly well known that when a baby is put to nurse at the mother's breast shortly after birth, the involution of the uterus is accelerated, and there is a resulting significant reduction in bleeding.

Lóránd and Asbót have recently shown that slight stimulation of the nipples in pregnant, parturient, and nursing women, results in a marked increase in the contractions of the uterus. Near term the usually experienced slight spontaneous contractions double in intensity, last longer, and continue for some time after the stimulus has been discontinued. In most of the parturients the contractions are only moderately increased. In the nursing woman the contractions are extremely strong, often as painful as during labor.

The benefits which the nursing infant receives both from the sucking and perioral stimulation, are seen in the comparative rarity of gastro-intestinal disorders in the breast-fed baby as compared with the bottle-fed baby. Further investigation will probably reveal that the breast-fed baby is a respiratorily more efficient organism than the bottle-fed baby.

Human beings do not lick their young, but in all cultures a certain amount of caressing is normally given the infant by the mother. Breast feeding brings the infant into close physical contact with the mother resulting in much labial and facial tactile stimulation in cultures where infant and mother wear clothes,[1] or total body stimulation where infant and mother habitually remain nude. It is possible that such cultured differences in raiment or nudity in the nursing situation, altogether

[1] It is of interest to note here that towards the end of the eighteenth century Erasmus Darwin pointed out that our fondness for rolling hills and gently undulating landscapes was probably derived from our conditioning upon the cushion of our mother's breast. This also recalls the lines from *Psalm* 12 "I will lift up mine eyes unto the hills, / From whence cometh my help."

apart from other incidental nursing practices, have some effect upon the subsequent development of the infant. This is a subject which has thus far received no adequate study. However this may be, the fact is that in all human cultures physical contact between persons plays an important role in their interpersonal relations. Hand-shaking, nose-rubbing, kissing, putting one's arm around another person, holding hands, walking arm-in-arm, nuzzling, and the like, are evidences of affection or friendliness. Avoidance of such acts indicates the withholding of affection or social nonrecognition. In Western cultures it is still the practice of well-bred persons to apologize to a stranger whom they may accidentally have touched, and to do so even to a friend or close relative. To establish contact with another is an act of communication, of social recognition. If there has been no formal or other occasion for such social recognition the act is considered out of place.

Children want to touch everything. It is only gradually that they learn what and when they may or may not touch. And there is a strong tendency on the part of human beings to take nothing for certain unless they can touch it. They want "tangible" evidence.

Spurgeon English has stated that love and touch are inseparable and indivisible, that love cannot arise in the human being without touch and sensuous arousal, and that the cooperation necessary for social conformity is not possible without affection and tactile stimulation. This may be putting it extremely, but it is probably not far from the truth. What we love we want to touch. The supreme act of touching is the sexual act with its physiological relief of tension through orgasm.

Physical proximity insofar as it approximates tactile association tends toward social homeostasis. Separation tends to produce disequilibrium. As English says: "Misunderstandings which arise through wide separation are cleared up on close contact." When people "get together," "into touch," or "contact," they "stroke" each other the right way, when they remain apart they do so the wrong way. Quite obviously the modern tendency for husband and wife to occupy separate beds should be discouraged.

Charlotte Wolff points out that "In protective affection tenderness has a 'tactile' quality, expressed chiefly by cautious and subtle gestures of the hands which satisfy both the pleasure of contact and an unconscious physical curiosity. By touching the object of affection the child gains his first emotional and sensuous knowledge of others."

It is here being suggested that tactile stimulation during infancy, and especially during the first months of nursing, is extremely important for the subsequent development of the person. That inadequate tactile stimulation may retard and mar the development of the person in a variety of ways which, though not yet investigated or demonstrated, nevertheless can be conjectured. On the physical level it may be suggested that persons who have received insufficient tactile stimulation in infancy are frequently shallow breathers and are more susceptible to disorders of the gastro-intestinal and respiratory tracts and possibly the genito-urinary tract than those who have received an adequate amount of cutaneous stimulation. Behaviorally it may be that such persons are abnormally preoccupied with securing cutaneous stimulation in all sorts of ways. These are but conjectures about possible relationships which remain to be investigated. Schematically the need for cutaneous stimulation may be analysed as follows:

							Satisfaction	
Physiological Tension	=	Urge or Need to	→	Which Leads to the Act of	→	Homeostasis		
General Tension	=	be caressed	→	contact	→	soothing effect		

Is it possible that we need to caress, to massage, infants rather more than we have recognized as necessary in the past? If it is true that cutaneous stimulation is a way of communicating with the infant, should he not receive more reassurance through his skin than he has cutomarily been given? May it not be that if we massage his skin in infancy it may later be unnecessary for a physician to massage his psyche?

A great deal of research will have to be done before these questions can be answered. It is hoped that this discussion may be instrumental in stimulating such research. Meanwhile, there exists a certain amount of evidence which indicates that cuta-

neous stimulation in an adult who has had an inadequate amount of it in infancy, may relieve the person of a disorder which is, *ex hypothesi*, related to that inadequate cutaneous stimulation in infancy. The following report describes such a case.

Mrs. C.—., an Englishwoman, aged 30 years, divorced and childless, height 5 feet 4 inches, weight 90 lbs., seen in July, 1948, in London, England. One of identical twins. Both had suffered approximately fortnightly episodes of asthma since they could remember. For the last 6 years Mrs. C.— had been in and out of sanitaria for treatment. Her doctor had informed her that if she suffered another attack it might be her last. In calling upon her at her own home, it was noted that she looked quite healthy, pretty, and rather tense. She greeted her visitor with a cold, limp hand, and then folded her forearms over her chest, and sat down upon a davenport against which she immediately began rubbing her back, in a quiet and almost unnoticeable manner. To the question whether her mother had died when she was young, she replied when she was born, and asked how the inference had been made. It was explained from the following facts: (1) the way she had limply shaken hands, (2) by what she had done with her hands immediately afterwards, and (3) by the way she had scratched her back against the davenport. All this had suggested that she might not have received adequate cutaneous stimulation when she was an infant; and since this frequently came about as a result of the mother's death, the "obvious" inference was made. The theory of the relationship between cutaneous stimulation and the development of the respiratory system was discussed, taking particular pains to emphasize the fact that this was just a theory, and that there was nothing about it that had yet been proven, but that if she wished she might give it a try. It was suggested that she go to a physiotherapy clinic where, according to instructions, she would be massaged by an expert masseuse. To this she readily agreed, and several days later, after her first massage, she was overflowing with enthusiasm. She was told that the probabilities were high that if she continued the massages for some time, she would never have another asthmatic attack unless, possibly, she was involved in some critical emotional upset. She continued with the treatment for several months, and during the more than five years which have since elapsed she has been in the best of health.

Mrs. C.—'s sister had had identical attacks of asthma until she married a famous author, whereupon her attacks declined in frequency although they did not altogether cease. In Mrs. C.—'s case the asthmatic attacks have been reduced to a minimum. There may be little or no relation here between the relief and the cutaneous stimulation she received. The relief may

be entirely due to suggestion. This case has been cited for its suggestive value. It is to be hoped that those having the adequate opportunities may carry out the observations necessary to show whether or not any asthmatics may be relieved by a course of cutaneous stimulation given them on the theory outlined in the present paper.

Summary

Evidence is cited which suggests that the skin as an organ has a greater functional significance for the physiological and psychological development of the organism than has hitherto been commonly recognized.

The evidence indicates that in nonhuman mammals the licking of the young by the mother serves to activate such sustaining systems of the body as the genitourinary, the gastrointestinal, and the respiratory.

In man there is some evidence which indicates that a relation exists between cutaneous stimulation and the development of the sustaining systems of the body.

A case is reported of relief of a long-standing asthmatic condition in one of a set of identical twins who was treated by cutaneous massage.

REFERENCES

Drillien, M.: Studies in Prematurity. Part 4. Development and Progress of the Prematurely Born Child in the Pre-School Period. *Arch. Dis. Childhood*, 23: 69–83, 1948.

English, S.: Sex and Human Love, in *About the Kinsey Report* (edited by D. P. Geddes and E. Currie). New York, New American Library, 1948, pp. 101–102.

Hammett, F. S.: Studies of the Thyroid Apparatus. *Endocrinology*, 4: 221–29, 1922.

Lóránd, S., and J. Asbót: Ueber die durch Reizüng der Brustwarze angeregten reflektorischen Uteruskontraktionen. *Zentralbl. f. Gynäk.*, 74: 345–352, 1952.

Montagu, M. F. Ashley. Some Factors in Family Cohesion. *Psychiatry*, 7: 349–352, 1944.

Osler, William: *Aphorisms* (Collected by R. B. Bean and Edited by W. B. Bean). New York, Henry Schumann, Inc., 1950, p. 126.

Reyniers, J. A.: Germ-Free Life Studies. *Lobund Reports*, No. 1, 1946, and No. 2, 1949.

Shirley, M.: A Behavior Syndrome Characterizing Prematurely-Born Children. *Child Development*, 10: 115–28, 1939.

Windle, W. F.: *Physiology of the Fetus*. Philadelphia, Saunders, 1940.

Wolff, C.: *A Psychology of Gesture*. Methuen, London, 1948, p. 49.

CLINICAL MANAGEMENT OF THE IRRITABLE COLON AND DIVERTICULOSIS

JAMES W. MORGAN*

The medical advice offered to patients suffering from irritable colon or diverticulosis is usually quite inadequate and they are often confused rather than benefited by the oral or written instructions which they receive from their physicians. Although there may be agreement as to the mechanical, physiologic, chemical and bacteriological factors involved in these conditions, the management of these patients remains controversial. In the present state of our knowledge, permanent cure of these patients can only be anticipated following partial or complete excision of the colon. These surgical procedures are strictly limited to highly selected cases under special circumstances. Therefore, in the majority, effective therapy is the maintenance of an appropriate regimen and the physician's instructions will have to be followed over a period of many years if not a lifetime. Virtually the patient has to learn to live with his disease and consequently, the physician's instructions, human nature being what it is, should not be too strict.

Primarily, the management of the irritable colon revolves around the question of the control of the patient's diet. Yet, it is evident that the selection of a regimen is fundamentally dependent upon two factors, the necessity of maintaining an adequate nutrition and the necessity of avoiding all foods which irritate the colon. The welfare of the patient depends upon the establishment of a nice balance between these two factors within the framework of normal everyday living. The rigid dietary regimens often recommended fail by ignoring the social and physiological context of the patient's well-being and are, indeed, often harmful because of their nutritional inadequacy. Further, even where there is general agreement with respect to nutritional requirements, there is confusion as to what foods irritate the colon.

---

* From the Department of Proctology, St. Luke's Hospital, San Francisco.
Received for publication February 15, 1953.

It has become customary in cases of irritable colon to recommend fruit juices and to interdict the use of whole fruit. Fruit juices are recommended in the fond belief that they are harmless and provide a convenient source of ascorbic acid (vitamin C). Nevertheless, for the past ten years we have advised against the use of concentrated fruit juice in cases of irritable colon. Clinical evidence demonstrates that the fruit juices by themselves are highly irritable to both colon and rectum, presumably because of their high content in fruit sugars and acids and particularly since juices are always taken at the beginning of a meal. Further, it should be remembered that fruit juice contains less "C" than the whole fruit. In their irritating effect, there appears to be little or no difference between "fresh," canned or frozen fruit juice. There is no objection to the fruit juices when sufficiently diluted to act as flavoring to beverages such as lemonade and orangeade.

It is curious that whole fruit should have been interdicted in cases of irritable colon especially in view of their great value in bowel regulation and relatively innocuous effects. Braconnet has shown that protopectins which are present in fruit (in proportions of 6 to 1 as compared to coarsely strained fruit juice and 9 to 1 compared to fruit put through a 20 mesh screen) provide valuable nutritional benefits by their very presence in the intestinal contents and by their desirable regulatory effect upon intestinal pH. In addition they exert many other actions which contribute to the physiologic activity of the intestinal tract. After ingestion protopectin is converted to a colloidal solution of pectin. The pectin remains intact for a considerable period of time in the intestinal tract because it is not hydrolyzed by the salivary, gastric or intestinal enzymes or those elaborated by commonly occurring intestinal pathogens. On undergoing hydroloysis, pectins are either partially or entirely de-esterfied, or their molecular chains are ruptured in greater or lesser degree as shown by Werch and Ivy. On the other hand, Manville finds that enzymes of the microflora inhabiting the colon almost completely destroy pectin by hydrolysis, to the extent depending upon the length of time the pectin is exposed to these enzymes in the lower bowel. The chief products of this decom-

position are galacturonic, formic, acetic, and propionic acid. It is apparent that ingested protopectin undergoes a continuous series of changes from the stomach to the colon. Moro claims these products of hydrolysis exert a well defined, highly favorable influence upon intestinal function.

By increasing the bulk and fluid-retentive power of the upper intestinal contents, by giving them a smooth gelatinous consistency, and by lubricating the intestinal wall, pectin promotes normal peristalsis without any harsh mechanical irritating influence. The very presence of gelatinous bulk gently cleanses the mucosa of irritating effects. Because of their organic acid content, pectins lower the pH in the small intestine, thus helping, according to Werch, to suppress the growth of pathogens. In the colon the acids released by pectin are capable of exerting a bacteriostatic effect. Through the release of lower fatty acids and galacturonic acid ingested protopectins encourage growth of normal intestinal inhabitants. The consequent reduction of intestinal pH, harmless to the normal flora, inhibits growth of many putrefactive and otherwise undesirable micro-organisms present in the intestine. In addition, several investigators claim that galacturonic acid has a detoxifying influence within the bowel. Apparently with a continuous supply of protopectin, provided by the whole fruit, the physiologic state of the intestinal tract can be appreciably improved, and many commonly occurring digestive aberrations and functional derangements can be avoided.

All patients with diverticulosis or irritable colon are therefore urged to eat fruit (raw, cooked, canned or dried), vegetables and salads. Proper nutrition is vital. Details of the diets advocated will be found at the end of this article.

Medication is used to relieve definite symptoms. Appropriate sedation with atropine, belladonna, trasentin, donatal, phenobarbital, etc., is oftentimes indicated. There is one important warning. Mineral oil in any form or in any combination is by no means free of toxicity. It should never be taken as a laxative and in repeated doses over considerable periods of time, as commonly used in the treatment of constipation, serious cumulative toxic effects may occur. My own studies have shown that mineral oil is an irritant to the colon. In the

case of a ruptured diverticulum of the colon, it would seem to be far safer to have a hard fecolith (which might be sterile) free in the abdominal capity, than a quantity of dirty oily feces.

Proper Food for Irritable Colon (and Diverticulosis): The following foods are *never* permitted: corn, popcorn, celery stalks, bran in any form, strong condiments, Mexican and Italian peppers, hot curries and any food which is hard to digest or might adversely affect any individual. Alcoholic beverages in moderate quantities are permitted. These suggestions are recommended for all patients who have disorders of the lower intestine.

For those persons who have good digestion: At least two meals a day should include one or two generous servings of fruit, vegetables, and salads: *Fruits:* (Raw, cooked, canned or dried) oranges, grapefruit, tangerines, apples, apricots, prunes, peaches, cherries, plums, grapes, figs, bananas, pears, raisins, dates, and pineapples. *Vegetables:* Spinach, celery root, tomatoes, raw cabbage, cauliflower, Brussels sprouts, asparagus, turnips, parsnips, rutabagas, carrots, potatoes, string beans, squash, pumpkin, onions, artichokes, peas, cucumbers, and beets. *Salads:* Salad greens of all kinds, fruit salad, and vegetable salad, omitting celery stalks.

With this diet there should be an adequate amount of proteins from beef, lamb, veal, pork, chicken, fish or cheese. It is advisable to take at least a glass full of milk a day and at least one egg to supply the necessary minerals and vitamins. The taking of much milk is inadvisable because it often tends to produce hard, ovulated stools.

A smoother diet is suggested for persons who cannot digest rough foods without getting indigestion, gas and abdominal discomfort. This diet should contain as much fruit as possible. Fruits suggested are: Oranges, grapefruit, stewed prunes, applesauce, baked apples, stewed apricots, cooked pears, or peaches, and ripe bananas. Vegetables recommended are: Turnips, string beans, asparagus, beets, carrots, squash, peas, and creamed spinach. Any vegetable and fruit may be used if they have been pureed or prepared in a mechanical blender. Starches should include whole wheat or graham bread, crackers, crisp toast, rice, macaroni, noodles, spaghetti, and any cereal that does not contain bran. Salads are made with tomato aspic, tomatoes, asparagus, cottage cheese, pears, peaches, bananas, avocados, gelatin, and shredded lettuce. The following foods may be used as tolerated: Meat, fish, shellfish, crisp bacon, ham, eggs, cream, butter, coffee, tea, cocoa, and soups. Desserts consist of simple puddings made of bread, rice, tapioca, sago, custard, junket, gelatin, blanc mange, ice cream, simple cakes, canned fruit, the fillings of apple, peach, custard or lemon pie, and cornstarch, cottage cheese, and cream cheese.

position are galacturonic, formic, acetic, and propionic acid. It is apparent that ingested protopectin undergoes a continuous series of changes from the stomach to the colon. Moro claims these products of hydrolysis exert a well defined, highly favorable influence upon intestinal function.

By increasing the bulk and fluid-retentive power of the upper intestinal contents, by giving them a smooth gelatinous consistency, and by lubricating the intestinal wall, pectin promotes normal peristalsis without any harsh mechanical irritating influence. The very presence of gelatinous bulk gently cleanses the mucosa of irritating effects. Because of their organic acid content, pectins lower the pH in the small intestine, thus helping, according to Werch, to suppress the growth of pathogens. In the colon the acids released by pectin are capable of exerting a bacteriostatic effect. Through the release of lower fatty acids and galacturonic acid ingested protopectins encourage growth of normal intestinal inhabitants. The consequent reduction of intestinal pH, harmless to the normal flora, inhibits growth of many putrefactive and otherwise undesirable micro-organisms present in the intestine. In addition, several investigators claim that galacturonic acid has a detoxifying influence within the bowel. Apparently with a continuous supply of protopectin, provided by the whole fruit, the physiologic state of the intestinal tract can be appreciably improved, and many commonly occurring digestive aberrations and functional derangements can be avoided.

All patients with diverticulosis or irritable colon are therefore urged to eat fruit (raw, cooked, canned or dried), vegetables and salads. Proper nutrition is vital. Details of the diets advocated will be found at the end of this article.

Medication is used to relieve definite symptoms. Appropriate sedation with atropine, belladonna, trasentin, donatal, phenobarbital, etc., is oftentimes indicated. There is one important warning. Mineral oil in any form or in any combination is by no means free of toxicity. It should never be taken as a laxative and in repeated doses over considerable periods of time, as commonly used in the treatment of constipation, serious cumulative toxic effects may occur. My own studies have shown that mineral oil is an irritant to the colon. In the

case of a ruptured diverticulum of the colon, it would seem to be far safer to have a hard fecolith (which might be sterile) free in the abdominal capity, than a quantity of dirty oily feces.

Proper Food for Irritable Colon (and Diverticulosis): The following foods are *never* permitted: corn, popcorn, celery stalks, bran in any form, strong condiments, Mexican and Italian peppers, hot curries and any food which is hard to digest or might adversely affect any individual. Alcoholic beverages in moderate quantities are permitted. These suggestions are recommended for all patients who have disorders of the lower intestine.

For those persons who have good digestion: At least two meals a day should include one or two generous servings of fruit, vegetables, and salads: *Fruits:* (Raw, cooked, canned or dried) oranges, grapefruit, tangerines, apples, apricots, prunes, peaches, cherries, plums, grapes, figs, bananas, pears, raisins, dates, and pineapples. *Vegetables:* Spinach, celery root, tomatoes, raw cabbage, cauliflower, Brussels sprouts, asparagus, turnips, parsnips, rutabagas, carrots, potatoes, string beans, squash, pumpkin, onions, artichokes, peas, cucumbers, and beets. *Salads:* Salad greens of all kinds, fruit salad, and vegetable salad, omitting celery stalks.

With this diet there should be an adequate amount of proteins from beef, lamb, veal, pork, chicken, fish or cheese. It is advisable to take at least a glass full of milk a day and at least one egg to supply the necessary minerals and vitamins. The taking of much milk is inadvisable because it often tends to produce hard, ovulated stools.

A smoother diet is suggested for persons who cannot digest rough foods without getting indigestion, gas and abdominal discomfort. This diet should contain as much fruit as possible. Fruits suggested are: Oranges, grapefruit, stewed prunes, applesauce, baked apples, stewed apricots, cooked pears, or peaches, and ripe bananas. Vegetables recommended are: Turnips, string beans, asparagus, beets, carrots, squash, peas, and creamed spinach. Any vegetable and fruit may be used if they have been pureed or prepared in a mechanical blender. Starches should include whole wheat or graham bread, crackers, crisp toast, rice, macaroni, noodles, spaghetti, and any cereal that does not contain bran. Salads are made with tomato aspic, tomatoes, asparagus, cottage cheese, pears, peaches, bananas, avocados, gelatin, and shredded lettuce. The following foods may be used as tolerated: Meat, fish, shellfish, crisp bacon, ham, eggs, cream, butter, coffee, tea, cocoa, and soups. Desserts consist of simple puddings made of bread, rice, tapioca, sago, custard, junket, gelatin, blanc mange, ice cream, simple cakes, canned fruit, the fillings of apple, peach, custard or lemon pie, and cornstarch, cottage cheese, and cream cheese.

The following suggestions are for all irritable colon patients: Drink no fruit juice. In drinking concentrated fruit juice (orange juice, grapefruit juice, pineapple juice, prune juice, etc.) one loses some of the benefit of the pulp which furnishes nonirritating bulk and retains moisture that is carried to the colon. Fruit juice alone seems to irritate both colon and rectum. Eat fruit. Vegetable juice (tomato juice, V-8) is permitted.

The body's need for water is determined by the individual's thirst. Excess water only means more work for the heart and kidneys. A glass or two of hot water, each with one-third teaspoon of salt, before breakfast is oftentimes beneficial, if no salt or sodium restriction has been advised for the patient by his doctor. This (sometimes) "cures" constipation. Normal saline enemas (*level teaspoon of salt* to *one* pint of warm water) (exact measurement) can safely be taken every day. For those who have severe constipation or those who think they need laxatives, a daily saline enema is strongly recommended. Mineral oil in any form should never be taken as a laxative. Bran is not meant for human consumption.

REFERENCES

Braconnot, Henri: New Observations on Pectic Acid. *Ann. Chim. Phy.*, 30: 96–102, 1925.

Manville, I. A., E. M. Bradway, and A. S. McMinis: Pectin as a Detoxication Mechanism. *Am. J. Digestive Disease Nutrition*, 3: 570–72, 1936.

Morgan, J. W.: Misgivings of Mineral Oil as a Laxative. *Am. J. Surg.*, 92: 360–364, 1938.

Morgan, J. W.: The Harmful Effects of Mineral Oil Purgatives. *J.A.M.A.*, Vol. 117: pp. 1335–1336, 1941.

Moro, E.: Two Day Apple Diet for Treatment of Diarrheal Conditions in Childhood. *Klin. Wochschr.*, 8: 2414–17, 1929.

Werch, S. C., R. W. Jung, H. Plenk, A. A. Day, and A. C. Ivy: Pectin and Galacturonic Acid and the Intestinal Pathogens. *Am. J. Disease of Children*, 63: 839–46, 1924.

Werch, S. C., and A. C. Ivy: On the Fate of Ingested Pectin. *Am. J. Digestive Disease*, 8: 101–105, 1941.

THERMAL STRESS AND TISSUE CULTURE

I. The Effect of Rapid Freezing and Thawing on Chick Embryonic Epidermis, Skeletal Muscle and Spindle Cells*

C. M. Pomerat and Robert B. Lewis

It has been demonstrated that *in vivo* all tissues do not show the same degree of susceptibility to local cold injury (Pichotka, Lewis, and Freytag, 1951; Lewis, 1951; Friedman, Lange, and Weiner, 1950; Lewis and Moen, 1952). Muscle and nerves are quite easily damaged whereas skin and tendon are more resistant. These same investigations indicate that the attained tissue temperature is not the all important factor in producing damage, but rather the product of temperature and exposure time. The observations of Lake (1917) namely, that —6° C. was critical for tissue survival, either isolated or *in vivo*, is still quoted in most fundamental cold studies on bodily tissues. More observations on this problem are necessary to determine the validity of this conclusion.

This investigation was undertaken to determine the effect of various cold temperatures on tissue culture growth when the time of exposure was reduced to a practicable minimum.

Materials and Methods. In typical experiments, 20 fragments each of 4 different species of tissue were transferred into 2 ml. of Gey's balanced salt solution in a thin-walled conical 15 ml. centrifuge tube. In some series, cockerel serum was substituted for the salt solution. An enamelized thermocouple made of 30-gauge copper-constantan wire was sterilized by immersion in 95 per cent ethanol for 2–3 minutes, washed with balanced salt solution and fixed in the centrifuge tube so that its tip

* From the Tissue Culture Laboratory, Medical Branch, The University of Texas, Galveston, and Department of Pathology, USAF School of Aviation Medicine, Randolph Field. This study was supported by funds provided under Contract AF 41(607)-36 with the USAF School of Aviation Medicine, Randolph Field. Grateful acknowledgment is made for indispensable aid with tissue culture procedures to Mrs. John Finerty and Mrs. Frank Dunton, to Mr. George Lefeber and Mr. Curtis Hoskins for photomicrographic records and to Mr. McDonald Smith for aid in the preparation of the graphs. Received for publication February 23, 1953.

rested in the center of the core of tissue fragments. After recording room conditions on a single point thermograph (Honeywell) from the thermocouple among the tissue fragments, the centrifuge tube was plunged into a mixture of 95 per cent ethanol and solid CO_2 at a temperature of approximately —70° C. As soon as the appropriate drop in temperature was achieved, the centrifuge tube was quickly transferred to a water bath maintained at +42° C. Usually the temperature continued to fall for some 2–3° C. during the transfer to the warm environment. Experiments were repeated until the desired range of low temperatures had been explored. A diagram illustrates the setup used for the freezing and thawing of the tissues (Fig. 1). Soon after the tissues had been warmed to 37° C., they were removed from the conical tube to a watch glass containing fresh Gey's solution and cultures were immediately prepared.

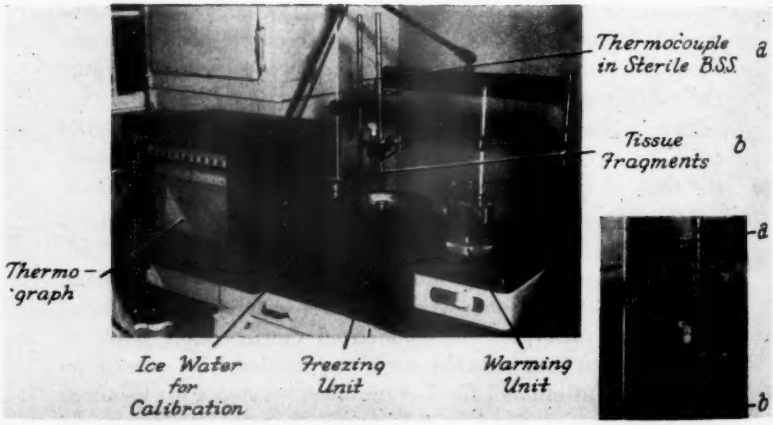


FIG. 1. Setup for freezing and thawing tissue explants before their transfer to culture media. Inset shows tubes containing sterile thermocouple (a) and the tissues in balanced salt solution (b).

It was generally found possible to make an accurate separation of the mixed fragments of the various tissue species on the basis of their form, color, the presence of young feathers or other characteristic features. For each tissue employed, 5

fragments were transferred onto 3 drops of heparinized cockerel plasma which had been placed on a cover glass of No. 1 thinness measuring 12 x 50 mm. To this, 3 drops of chick embryonic extract were added, mixed and the fragments evenly spaced on the slide. When the medium had become firmly clotted, two such slides were placed back-to-back in the lumen of Pyrex test tubes measuring 16 x 150 mm. and closed with a sterile cotton plug. One hour later 2 ml. of a fluid nutrient was added to each tube which consisted of 50 per cent human ascitic fluid (of malignant origin), 45 per cent Gey's balanced salt solution, 5 per cent chick embryonic extract and a quantity of penicillin sufficient to make 1,000 units per ml. The tubes were closed with No. 0 sterile rubber stoppers and placed in a culture-tube drum which rotated at 8 revolutions per hour in an incubator maintained at 37.5° C.

Outgrowth could usually be seen by examining living cultures within the test tubes by using a 32 mm. objective and a 10 x ocular on a compound microscope. All cultures in each group of experiments were fixed after 2, 3 or 6 days with absolute methyl alcohol and stained by Jacobson's method (Giemsa, followed by the May-Grünwald mixture). Slides were examined primarily for the presence of cells showing viability as revealed by their capacity to emigrate from the explants. This was also evident as a result of changes in cell form.

While every effort was made to obtain nutrients under standardized conditions, it was realized that the constituents of the basic plasma clot, of the fluid nutrient medium, and of the test tissues may show some degree of variation from experiment to experiment. In the methodology of quantitative studies involving tissue culture techniques, it is therefore considered wise to repeat experiments with media components (in this series, heparinized rooster plasma, chick embryonic extract, and human ascitic fluid of malignant origin) obtained at various times in order to assure the reproducibility of results. Series of cultures are reported together according to their experiment number so that the homogeneity of the sampling may be assured and sufficient cross-information is available to assure the reliability of the interpretations. For these reasons, protocols and

composites of temperature graphs are presented which have formed the bases for the summaries of collected information.

In the first series of experiments damaged tissues were cultivated for only 48 and 72 hours but, because of the excellent outgrowth of epithelium and of multinuclear straps of skeletal fibers obtained with longer periods of incubation, a 6-day limit was set. However, for this report it was thought desirable to include data obtained after less than 6 days' incubation in an effort to discover whether recovery from cold injury was progressively enhanced with time.

Mechanical disturbance and liquefaction of the unevenly thin portions of the plasma clot occasionally caused some explants to become dislodged from the culture slides. Results are presented, therefore, in terms of the total number of explants which persisted during the period of incubation. Thus, a report for positive epithelial outgrowth rated at 12/19 refers to the fact that out of 19 explants available for study in final stained preparations only 12 showed emigrating epithelium.

Results

Muscle was identified as elongated straps containing several nuclei (Figs. 9–16). In young, vigorous cultures the nuclei generally were arranged in a single row while older cultures or those showing injury often were found to have from two to four rows of nuclei. Such fibers often displayed the presence of characteristic cross striations both in living and stained preparations (Figs. 15, 16) and showed rhythmic spontaneous contractile activity. The description of the movement of isolated skeletal fibers in the outgrowth of chick embryonic leg muscle is deserving of much further study. Untreated control cultures incubated for 6 days frequently exhibited aggregations of nuclei at the distal end of the sarcoplasmic band and occasionally giant cells could be found (Figs. 12, 13, 14). These phenomena, which were also used as criteria of muscular injury in explants having been exposed to temperature stress, were compared within a given series of experiments to evaluate the degree of damage produced.

Epidermal cells were identified as flattened elements occurring in sheets with intimate contact with their fellows at the cytoplasmic margin (Figs. 28, 29). Phase contrast, time-lapse cinematography reveals that active movements of the free edge of the outgrowing colonies are characteristic of this type of tissue.

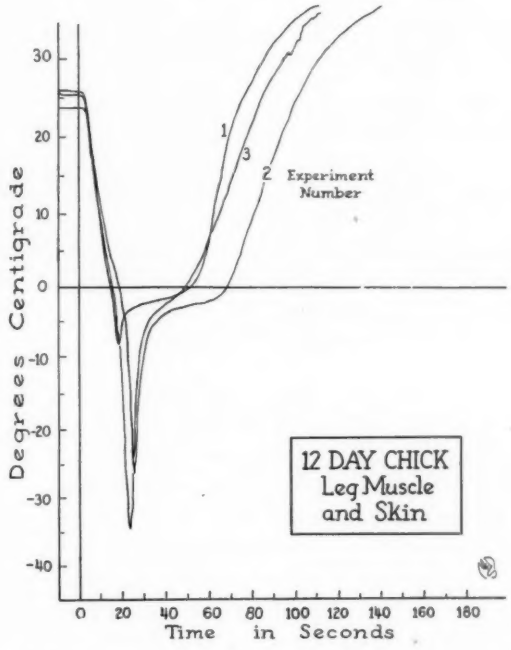


FIG. 2. Thermographic records of freezing and thawing for Experiments 1–3.

While the fusiform cells described in this paper probably generally represented elements of the fibroblastic type, it was considered more generic to refer to these as "spindle" cells. It is recognized that fibroblastic transformation *in vitro* may result from changes in a wide variety of cell strains. On the basis of Parker's (1933) demonstration that the growth rate

of fibroblasts from various organs appears to be relatively specific and constant, it became important to determine whether additional evidence for the concept of "physiological races" of spindle cells would be found in terms of differential resistance to the injurious effect of exposure to cold.

A. Leg Muscle

1. *Experiments 1–3.* 12-day chick tissue cultivated for 2 days. Temperature records in figure 2.

Very little evidence of muscle fiber formation and emigration was found in the outgrowth from these cultures (Fig. 3). However, in some areas showing aggregations of elongated cells, considerable mitotic activity and beginning multinucleations were suggestive of myogenesis (Fig. 4). Cold injury reduced the amount of outgrowth but a few spindle cells were found even after attaining a temperature of —35.4° C. in 25 seconds (Figs. 5, 6). These appeared to be emerging from the explants at points where blood vessels had been cut.

2. *Experiments 4–6.* 13-day chick tissue cultivated for 2 days. Temperature records in figure 7-A.

While these explants were obtained from chicks only one day older than those of the previous series, there was a considerably greater amount of outgrowth (Fig. 7) and degree of muscle differentiation. Straps of multinuclear muscle cells still were numerous following freezing to —9.0° C. in 22 seconds (Fig. 8). With freezing to —10.4° C. (in 25 seconds), however, the muscle tissue appeared to have been badly damaged.

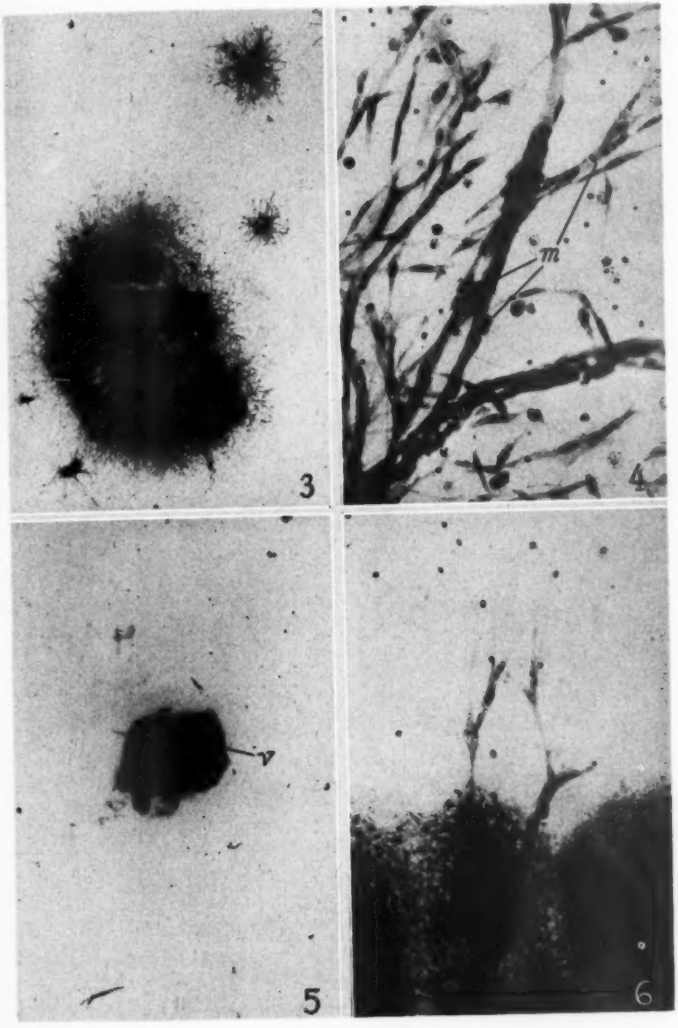
FIGS. 3–6. Cultures from Experiments 1–3 of 12 day chick embryonic leg muscle after 2 days *in vitro*.

FIG. 3. A control culture which shows typical spindle cell outgrowth. 12 x.

FIG. 4. The same as figure 3 showing multinuclear muscle fiber formation at 250 x. Note the presence of mitoses indicated by the letter *m*.

FIG. 5. Fragment of muscle after exposure to a temperature of —35.4° C. produced in 25 seconds. A few spindle cells emigrated from the region of a vessel in the explant at the point indicated by the letter *v*. 12 x.

FIG. 6. Same as figure 5 but showing spindle cell outgrowth at 250 x.


3
m
4
v
5
6

3. *Experiments 7–8 and 9–10.* 11-day chick tissue cultivated for 6 days. Temperature records in figure 11.

Even though the explants were younger than those used in previous series, the incubation of the cultures for 6 rather than

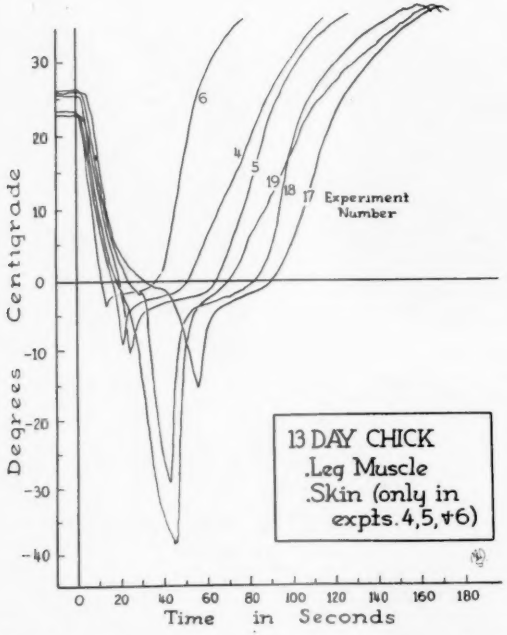


FIG. 7-A. Thermographic record of freezing and thawing for Experiments 4–6 and 17–19.

FIGS. 7–8. Cultures from Experiments 4–6 of 13 day chick leg muscle after 2 days *in vitro*. Both at 12 x.

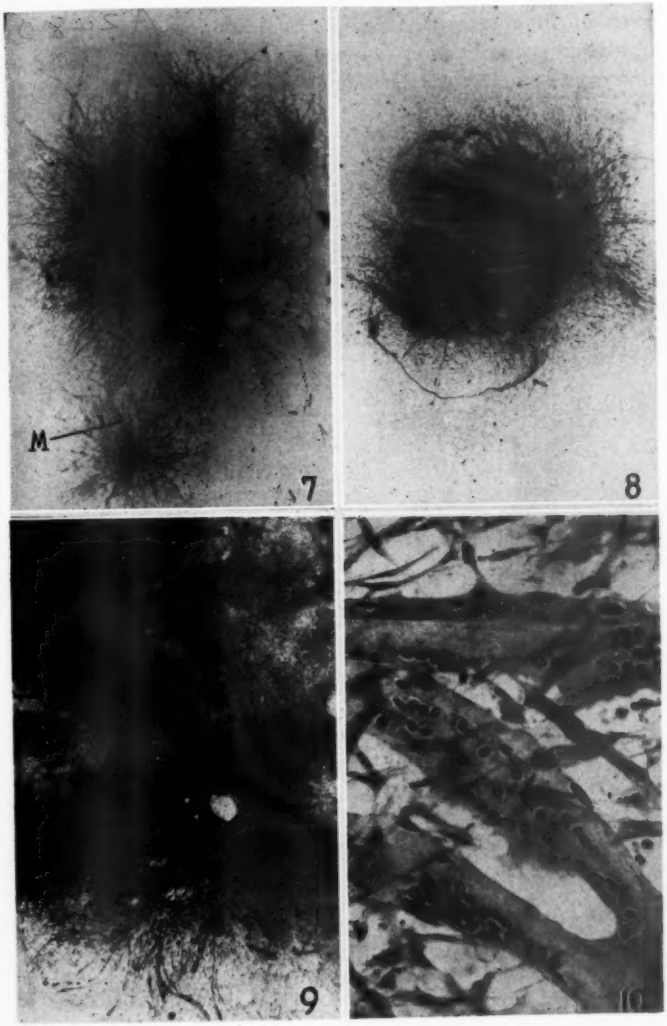
FIG. 7. A control culture showing spindle cell overgrowth and muscle fibers (indicated by letter *M*).

FIG. 8. Culture of fragment exposed to —9.0° C. in 22 seconds. Excellent muscle fiber outgrowth (M).

FIGS. 9–10. Cultures from Experiments 7 and 8 of 11 day chick leg muscle after 6 days *in vitro*.

FIG. 9. Outgrowth from a typical control culture showing muscle fibers especially well in the lower part of the figure. 12 x.

FIG. 10. Multinuclear muscle strap from the same culture as shown in figure 9 but at 250 x.


M
7
8
9
10

2 days resulted in the formation of well differentiated muscle fibers in the outgrowth (Fig. 9). Examination of such cultures at higher magnification showed muscle straps containing as many as 4 rows of nuclei (Fig. 10). Variations in form of the

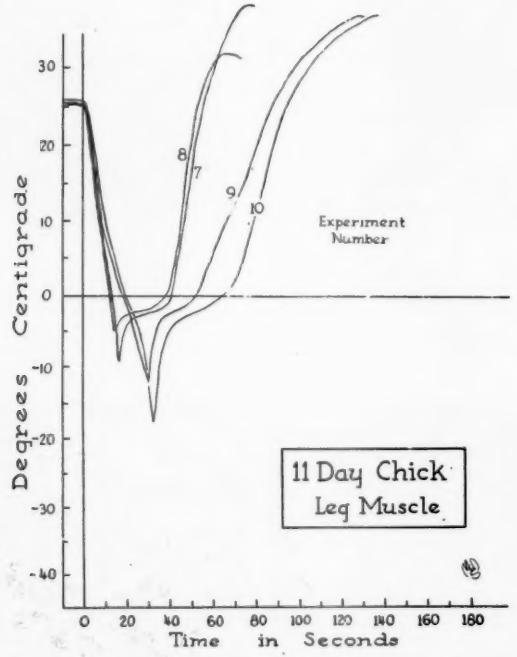


FIG. 11. Thermographic record of freezing and thawing for Experiments 7–8 and 9–10.

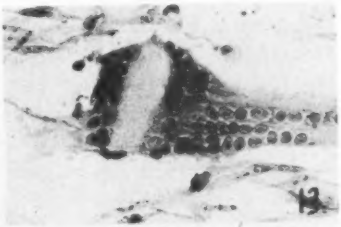
FIGS. 12–14. Control culture of 11 day leg muscle—6 day *in vitro* from Experiments 7 and 8 showing variations in the structure of multinuclear straps enlarged from same culture as that represented in figure 9. 250 x.

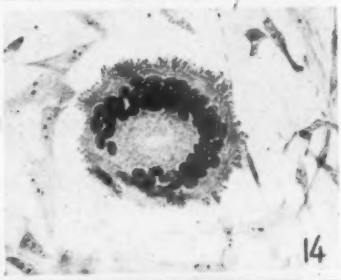
FIGS. 15–16. Muscle fibers from cultures comparable with those represented in figures 12–14. These are the only two photomicrographs of cells which were not stained by Jacobson's method in this series of illustrations. 1140 x.

FIG. 15. Living muscle fiber showing striations with dark phase contrast microscopy.

FIG. 16. Striations in a muscle fiber demonstrated by Bodian's protargol silver method.

12


13


14

15

16

muscle fibers included the presence of nuclei extending beyond the general line of the margin of the sarcoplasm (Fig. 12), terminal expansions with nuclear aggregation (Fig. 13) and giant cell formation (Fig. 14). A muscle fiber of similar type represented in figure 15 shows the arrangements of striations in the living culture as revealed with phase contrast optics, while figure 16 was obtained after treatment with Bodian's protargol method. Muscle fibers of this type are seen to exhibit rhythmic contractions even when almost completely isolated in the zone of outgrowth. The analysis of this behavior lies outside of the present paper.

In this series of experiments, muscle fibers resisted marked injury following exposure to a temperature of —11.9° C. obtained in 32 seconds (Expt. 9). A typical culture is shown in figure 17. While muscle was certainly damaged, a heavy outgrowth of spindle cells was obtained as shown in figure 18 after a drop to —18.2° C. in 34 seconds (Expt. 10).

4. *Experiments 17–19.* 13-day chick tissue cultivated for 3 days. Temperature records in figure 7-A.

This series which serves as a rechecking experiment shows again the importance of the 6-day time factor, although even after 3 days' incubation muscle fibers were obtained (Fig. 19). Muscle outgrowth was reduced after exposure to —15.6° C. in 58 seconds (Expt. 17) but evidence was again obtained for the viability of spindle cells at temperatures below —25.0° C. Figure 20 at low power and figure 21 at high magnification

---

FIGS. 17–18. Cultures in the same series as shown in figures 9–14. 12 x.

FIG. 17. Outgrowth after freezing to —11.9° C. in 32 seconds. Muscle fibers represented by the symbol *M*.

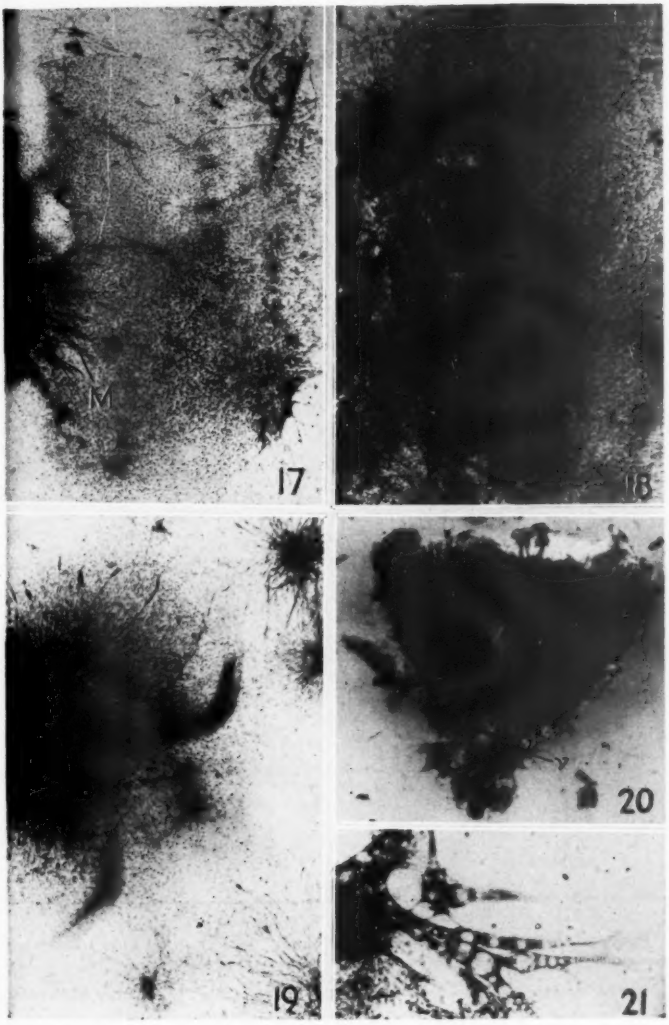
FIG. 18. Outgrowth from a tissue fragment after exposure to —18.2° C. induced in 34 seconds.

FIGS. 19–21. Cultures from Experiments 17–19 of 13 day chick leg muscle *in vitro* for 3 days.

FIG. 19. Control explant showing spindle cell and muscle fiber outgrowth. 12 x.

FIG. 20. Culture after exposure to —29.8° C. achieved in 43 seconds. A few spindle cells emigrated from a vascular area within the explant indicated at the point marked *v*. 12 x.

FIG. 21. The same culture as in figure 20 but at 250 x showing vacuolated spindle cells.


17
18
19
20
21

show results obtained in a culture exposed to —29.8° C. in 43 seconds. Fiber emigration showing marked injury evidenced by vacuolation was again seen to be associated with a vascular bed. Some spindle cells survived after freezing at —39.2° C. (Expt. 19).

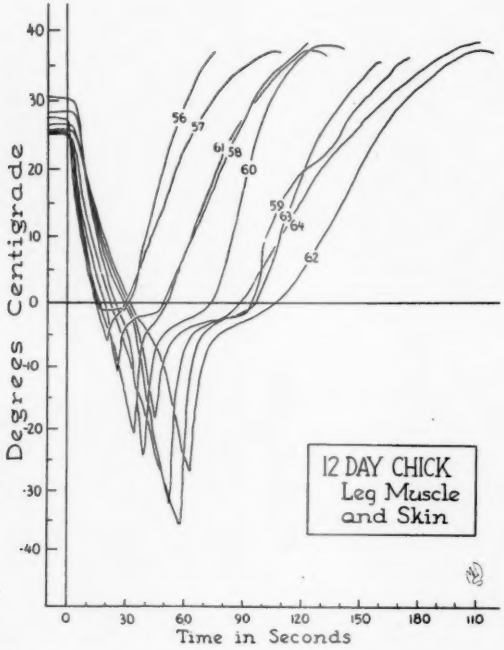


FIG. 22. Thermographic record of freezing and thawing for Experiments 56–64.

FIGS. 23–26. Cultures from Experiments 56–64 of 12 day chick leg muscle 6 days *in vitro*.

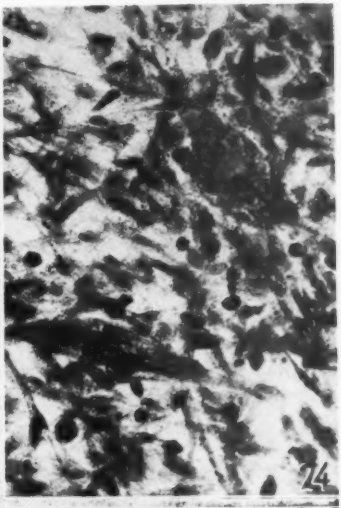
FIG. 23. Outgrowth from a control culture. Muscle fibers indicated by the symbol *M*. 12 x.

FIG. 24. The same as figure 23 but at 250 x showing muscle straps and a giant cell.

FIG. 25. Outgrowth from a fragment cultured after exposure to —5.9° C. in 24 seconds. 12 x.

FIG. 26. Fragment frozen to —20.5° C. in 35 seconds. 12 x.

M
23


24

25

26

5. *Experiment 32.* 13-day chick tissue cultivated for 6 days.

This experiment, which belongs to another series of studies in progress with different goals, is included because it illustrates the persistence of muscle fibers even after exposure to —20.6° C. in 29 seconds. While the temperature record is not presented, it belonged to the type characterized by rapid freezing and thawing.

6. *Experiments 56–64.* 12-day chick tissue cultivated for 6 days. Temperature records in figure 22.

This series of experiments was characterized by a less luxuriant outgrowth of muscle fibers than would have been expected in terms of the tissue material employed and the period of incubation. Unfortunately, an ascitic fluid of a different origin had to be used and it is believed that it was a less satisfactory nutrient. However, as seen in figures 23 and 24, typical

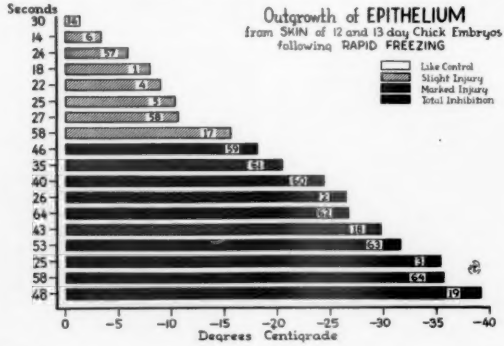


FIG. 27. Summary of results obtained from the cultivation of muscle fragments after cold injury. The time required to produce freezing temperature is represented in seconds at the left-hand margin of the graph.

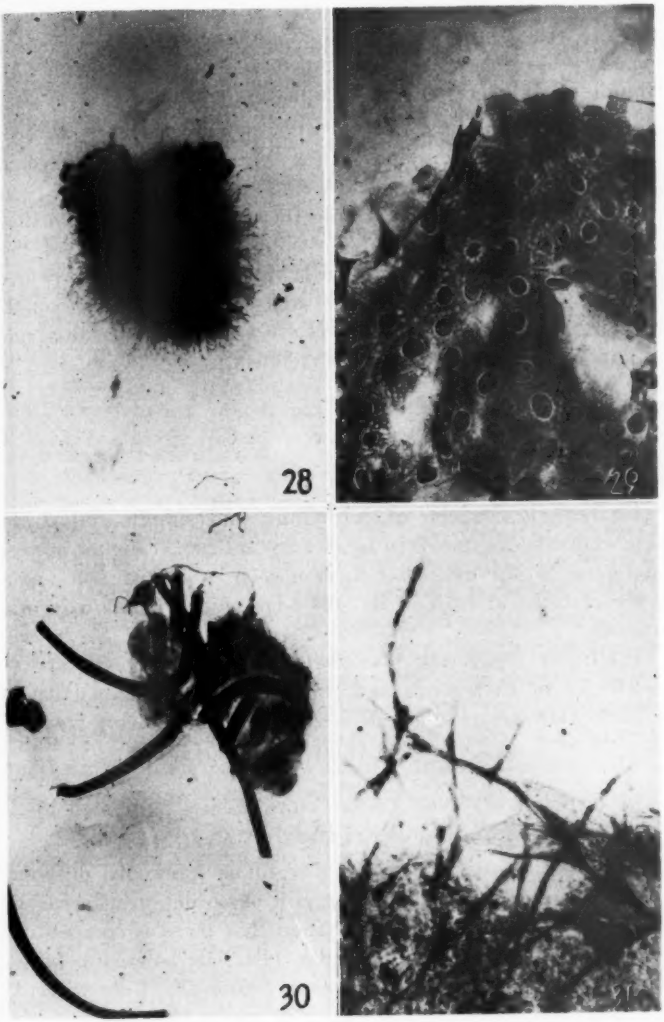
FIGS. 28–31. Cultures from Experiments 1–3 of chick embryonic skin after 2 days *in vitro*.

FIG. 28. Control culture showing both epithelium and spindle cells. 12 x.

FIG. 29. Same as figure 28 but at 250 x showing single layer of epithelial cells including 3 mitoses.

FIG. 30. Outgrowth from a fragment exposed to freezing at —35.4° C. in 25 seconds. Spindle cell emigration indicated by an arrow. 12 x.

FIG. 31. Same as figure 30 but at 250 x showing spindle cells.


28
29
30

muscle straps and small giant cells developed in control cultures. Marked injury to muscle was observed (Fig. 25) after exposure to only —5.9° C. obtained in 24 seconds (Expt. 57). Spindle cells were almost completely eliminated (Fig. 26) following freezing at —20.5° C. in 35 seconds (Expt. 61).

Summary. Results obtained for injury to chick embryonic leg muscle following rapid freezing and thawing have been summarized in figure 27. While there are some irregularities in the gradation of injury effects, it would appear that the critical level for the elimination of muscle fibers probably lies somewhere between —18.0° C. and —21.0° C. under conditions in which these temperatures are achieved in less than one minute and thawing immediately follows.

B. Skin

1. *Experiments 1–3.* 12-day tissue cultivated for 2 days. Temperatures recorded in figure 2.

Characteristic sheets of epithelium and spindle cells from skin explants are shown in figures 28 and 29. At higher power, in figure 29, the epithelial sheet is seen to consist of a single layer of cells, at least at its free edge, and to contain several mitoses.

Epithelial outgrowth was reduced after skin exposure to —8.0° C. in 18 seconds and, while totally eliminated below —25.0° C., rare spindle cells were observed following freezing at —35.4° C. in 25 seconds (Figs. 30 and 31).

2. *Experiments 4–6.* 13-day chick tissues cultivated for 2 days. Temperature records in figure 7-A.

Control cultures did not appear to be markedly different from those of 12-day chicks. Careful examination of series of such cultures leads to the conclusion that there is competition between the epithelial and spindle cells. The balance between the two species, such as is shown in figure 28, is not the rule but generally there is a dominance of one or the other.

Since this group of experiments did not include temperatures below —10.4° C., epithelium was not found to be damaged more than what can be described as slight injury.

3. *Experiments 17–19.* 13-day chick tissues cultivated for 3 days. Temperature records in figure 7-A.

Controls showed characteristic epithelial outgrowth in 15 out of 18 cultures. Feathers were well developed but these did not appear particularly disturbing to cellular emigration.

Experiment 18 was remarkable in that it showed typical epithelium in the outgrowth after exposure to —29.8° C. in 43 seconds. These results are represented at low magnification in figure 32 and at high in figure 33. Spindle cells were present after freezing at —39.2° C. but the epithelium seen in 3 out of 20 cultures did not appear to represent emigration but merely survival within the explant.

4. *Experiments 56–64.* 12-day chick tissues cultivated for 6 days. Temperature records in figure 22.

Epithelial outgrowth was obtained in all cultures in the control series. Only slight damage resulted from exposure to —10.7° C. in 27 seconds (Expt. 58) as shown in figure 34. While some epithelial outgrowth was seen in Experiment 61 with freezing to —20.5° C. in 35 seconds, none was found at —18.1° C. (Expt. 59). As discussed in the section dealing with muscle viability in that culture series, cellular emigration appeared to be less than would be expected on the basis of previous data. This may also account for the fact that spindle cells were not here found to survive exposure below —25.0° C. Figure 35 represents spindle cells in a culture from Experiment 61 in which freezing to —20.5° C.was achieved in 35 seconds.

Summary. The response of chick embryonic skin to cold injury in these experiments is summarized in figure 36. While total inhibition of epithelium was observed in several experiments with freezing of between —18.0° C. to —26.0° C., with one exception these belonged to the set numbered from 56 to 64 which was characterized by relatively poorer outgrowth of embryonic cells than would have been predicted in terms of previous experience. Moreover, since this may be attributable to the use of an ascitic fluid somewhat deficient in nutrient quality, it is believed that considerable weight should be placed upon the results obtained in Experiment 18 in which epithelial

outgrowth was seen in 8 out of 20 cultures from explants which had been exposed to freezing at —29.8° C. It is believed that the critical temperature for epithelial survival probably lies near this value.

C. Spindle Cells

An examination of the protocols indicates that spindle cells from skin are more resistant to cold injury than those derived from explants of leg muscle. This is well illustrated, for example, in Experiments 17 (—15.6° C.), 61 (—20.5° C.), 2 (—26.5° C.), 18 (—29.8° C.), and 3 (—35.4° C.).

As indicated by figures 6 and 21, spindle cells persisting after exposure to temperatures below —25.0° C. generally appeared to be associated with vascular tissue. Elongated elements emigrated in direct line with vessels which could be seen in the explants. Probably these did not represent the derivatives of endothelial cells whose free tips are characterized by sprays of threadlike points but may have originated from primitive adventitial elements.

These findings add evidence to Parker's (1933) observations that races of fibroblasts can be demonstrated by their growth potentialities in tissue culture.

Discussion. In the classic report on the effect of cold on explants cultivated *in vitro*, Lake (1917) employed hanging drop preparations. Many tissues, particularly young or embryonic heart fragments, from several species were studied. The culture medium generally consisted of homologous hypotonic plasma. It is not clear whether he employed embryonic extract. In

---

FIGS. 32–33. Cultures from Experiments 17–19 of chick embryonic skin after 3 days *in vitro*. The tissue fragment shown was exposed to a temperature of —29.8° C. in 43 seconds.

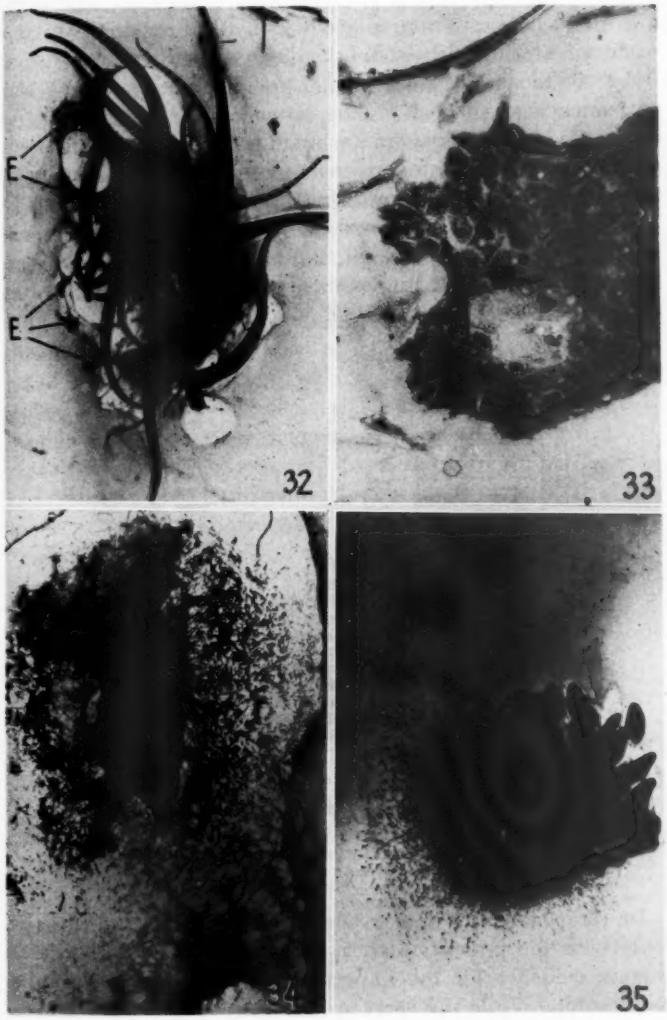
FIG. 32. Epithelium is indicated by the symbol *E*. 12 x.

FIG. 33. Same as figure 32 but at 250 x.

FIGS. 34–35. Culture from explants 56–64 of chick embryonic skin after 6 days *in vitro*.

FIG. 34. Epithelial and spindle cell outgrowth after freezing to —10.7° C. in 27 seconds. 12 x.

FIG. 35. Spindle cells and no evidence of epithelium after exposure to —20.5° C. obtained in 35 seconds. 12 x.


E
E
32
33
34
35

many of the experiments pulsatile activity and not outgrowth was used as the criterion of survival. Typical experiments consisted of storing tissues for 2 days at temperatures from +2.0° C. to storage at —10.0° C. for 30 minutes. There was no evidence of survival in the latter case. Actively growing tissues were treated in a similar manner. In these no outgrowth resulted after 30 minutes' exposure at —7.0° C. Lake concluded that his results closely approximated those obtained earlier by Lambert (1913) for chick and rat tissues. In both studies survival was markedly decreased at the level of —6.0° C. and in the graphs from both studies there was a dip in the curve at +14.0° C. to 16.0° C.

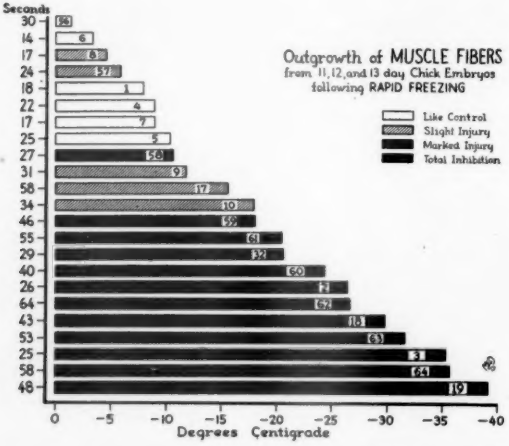


FIG. 36. Summary of results obtained following the cultivation of skin fragments after cold injury. The time required to produce freezing temperatures is represented in seconds at the left-hand margin of the graph.

In comparing the data reported by Lambert and also by Lake with the present work, it should be noted that there is further evidence for the importance of the time-temperature relationship. While the early studies were concerned with low temperatures imposed for as much as a half-hour, the data herein assembled was obtained under conditions of rapid freezing and thawing after the desired temperature had been reached.

Conclusions

1. Fragments of 11–13-day leg muscle and 12–13-day skin from chick embryos were subjected to freezing from —3.4° C. to —39.2° C. in less than one minute and then rapidly thawed in a water bath maintained at +42.0° C. All tissues were subsequently cultivated from 2 to 6 days in clots formed of cockerel plasma and chick embryonic extract in roller tubes. Viability was evaluated in terms of the amount of epithelial cells, muscle fibers and of spindle cells which emigrated as revealed in fixed preparations stained by Jacobson's method. Results were based on the study of 1,040 cultures.

2. In spite of some irregularities in the gradation of injury effects, the critical level below which muscle fibers did not emigrate lay between —18.0° C. and —21.0° C., while for epithelium the critical value was at approximately —30.0° C. Spindle cells from muscle explants appeared to be more vulnerable than those originating from the skin. A few spindle cells were found in the zone of outgrowth following freezing as low as —39.2° C. which are believed to represent elements from vascular structures.

REFERENCES

Friedman, N. B., K. Lange, and D. Weiner: Pathology of experimental immersion foot. *Arch. Path.*, 49: 21–26, 1950.

Lake, N. C.: An investigation into the effects of cold upon the body. *Lancet*, 2: 557–562, 1917.

Lambert, R. A.: The influence of temperature and fluid medium on the survival of embryonic tissues *in vitro*. *J. Exper. Med.*, 18: 406–411, 1913.

Lewis, R. B.: Pathogenesis of muscle necrosis due to experimental local cold injury. *Am. J. Med. Sc.*, 222: 300–307, 1951.

Lewis, R. B., and P. W. Moen: Experimental immersion leg. *Am. J. Med. Sc.*, 224: 529–539, 1952.

Parker, R. C.: The races that constitute the group of common fibroblasts: III. Differences determined by origin of explants and age of donor. *J. Exper. Med.*, 58: 401–414, 1933.

Pichotka, J., R. B. Lewis, and Ella Freytag: Sequence of increasing local cold injury. *Texas Rep. Biol. and Med.*, 9: 613–630, 1951.

Protocols for Experiments on Chick Embryonic Leg Muscle and Skin

	SKIN—12 day	LEG MUSCLE—12 day
CONTROL—2 day For Experiments 1–3	Excellent outgrowth of spindle cells. Epithelial sheets in 7/20.	Excellent outgrowth of loosely organized spindle cells in all cultures. Rare evidence of muscle fibers.
EXPERIMENT 1 —8.0°C. in 18 seconds	Relatively good outgrowth of spindle cells in 18/19 and epithelium in 2/19.	A few spindle cells in 9/20. No muscle.
EXPERIMENT 2 —26.5°C. in 26 seconds	Slight outgrowth of spindle cells in 8/19. No epithelium.	No evidence of outgrowth except a few spindle cells in one culture.
EXPERIMENT 3 —35.4°C. in 25 seconds	Rare spindle cells in 15/16. No epithelium.	Rare spindle cells in 9/20 but all appear to be derived from vessels in explants.

	SKIN—13 day	LEG MUSCLE—13 day
CONTROL—2 day For Experiments 4–6	Well-developed feather germs. Epithelium in 16/20 but suggestive of competition with spindle cells.	Heavy spindle cell outgrowth. A few narrow muscle straps in all cultures.
EXPERIMENT 4 —9.0°C. in 22 seconds	Poor spindles in 7/19. Epithelium in 4/19.	Spindle cells somewhat less rich but with slightly better development of multinuclear muscle straps.
EXPERIMENT 5 —10.4°C. in 25 seconds	Generally poor spindle cell outgrowth in 16/20 and epithelium in 8/20.	A few spindle cells in 6/18 and muscle fibers only in one culture.
EXPERIMENT 6 —3.4°C. in 14 seconds	A few spindle cells in 19/20. Epithelium in 4/20.	Outgrowth resembling control with some degree of greater flaring of the tips of muscle straps.

	LEG MUSCLE—11 day
CONTROL—6 day For Experiments 7 & 8	Excellent outgrowth of spindle cells. Numerous muscle fibers, generally slender but some with 3 rows of nuclei. Rare small giant cells.
EXPERIMENT 7 —9.°C. in 17 seconds	Resemble control but giant cells more common.
EXPERIMENT 8 —4.6°C. in 17 seconds	Like control but muscle fibers broader and giant cells somewhat more common.

Protocols for Experiments on Chick Embryonic Leg Muscle and Skin

	LEG MUSCLE—11 day
CONTROL—6 day For Experiments 9 & 10	Excellent outgrowth of spindle cells. Numerous multinuclear muscle straps some of which are very broad. Giant cells frequent.
EXPERIMENT 9 −11.9°C. in 32 seconds	Spindle cells less numerous. Muscle fibers not markedly different than those of controls except in some explants which show degeneration.
EXPERIMENT 10 −18.2°C. in 34 seconds	No marked evidence of damage. Some reduction of muscle cell emigration. A few spindle cells show pyknosis.

	SKIN—13 day	LEG MUSCLE—13 day
CONTROL—3 day For Experiments 17–19	Good spindle cell outgrowth. Epithelium in 15/18.	Excellent outgrowth of spindle cells. Numerous multinuclear muscle straps with broad ends.
EXPERIMENT 17 −15.6°C. in 58 seconds	Spindle cells like the control. Epithelium in 12/20.	Spindle cell and muscle fiber outgrowth somewhat reduced.
EXPERIMENT 18 −29.8°C. in 43 seconds	Spindle cells reduced. Some show vacuolation. Epithelium in 8/20.	Only a few spindle cells. These are usually associated with vessels in the explants and show vacuolation. No muscle.
EXPERIMENT 19 −39.2°C. in 48 seconds	Few spindle cells in 12/20.	Very few spindle cells in 3/20. No muscle.
CONTROL—6 day For Experiment 32		Excellent outgrowth of spindle cells. Numerous muscle fibers.
EXPERIMENT 32 −20.6°C. in 29 seconds		Some reduction of spindle cells. Broad basophilic muscle fibers and giant cells are numerous. Moderate injury.

Protocols for Experiments on Chick Embryonic Leg Muscle and Skin

	SKIN—12 day	LEG MUSCLE—12 day
CONTROL—6 day For Experiments 56–64	Good outgrowth of spindle cells. Epithelium in all cultures.	Good outgrowth of spindle cells. Moderate number of muscle fibers. Some giant cells.
EXPERIMENT 56 −1.4°C. in 30 seconds	Like control.	Like control but with somewhat more giant cell formation.

Protocols for Experiments on Chick Embryonic Leg Muscle and Skin

	SKIN—12 day	LEG MUSCLE—12 day
EXPERIMENT 57 —5.9°C. in 24 seconds	Considerable liquefaction. Spindle cell migration reduced. Epithelium in 8/16.	Spindle cell outgrowth less dense than control. Marked injury to muscle in 5/20.
EXPERIMENT 58 —10.7°C. in 27 seconds	Reduction in spindle cell outgrowth. Epithelium in 9/20.	10 cultures lost. Marked reduction in both spindle cells and muscle fiber outgrowth.
EXPERIMENT 59 —18.1°C. in 45 seconds	Spindle cells in only 1/20. No epithelium.	Spindle cells in only 1/20. No muscle.
EXPERIMENT 60 —24.4°C. in 40 seconds	No outgrowth.	Spindle cells in 1/20. No muscle.
EXPERIMENT 61 —20.5°C. in 35 seconds	A few spindle cells in 14/20. Damaged epithelial cells in only 1/20.	Spindle cells in 4/20. No muscle.
EXPERIMENT 62 —26.7°C. in 64 seconds	No outgrowth.	No outgrowth.
EXPERIMENT 63 —31.7°C. in 53 seconds	No outgrowth.	No outgrowth.
EXPERIMENT 64 —35.7°C. in 58 seconds	No outgrowth.	No outgrowth.

THERMAL STRESS AND TISSUE CULTURE

II. The Effect of Rapid Freezing and Thawing on Human Newborn and Adult Skin*

C. M. Pomerat and Robert B. Lewis

It is well known that the rate of emigration and of actual cellular proliferation of tissues derived from embryos is generally greater than that resulting from the explantation of fragments of corresponding adult animals. Observations on the effect of temperature on the viability of cells as measured with the technique of tissue culture usually have been based upon embryonic cells. It follows, therefore, that clinical interest in the basic factors resulting from frostbite and burn injury should include data obtained with the use of tissue explants from adult sources. While fur-bearing rodents probably can be relied upon to yield information which can be utilized in the interpretation of the physiology of human tissues subjected to stress, the desideratum of experimental studies on cold injury established directly upon adult human cells falls within the boundaries of reasonably attainable goals in modern tissue culture practice.

The object of the present study not only was to provide a comparison of the response of 11 to 13 day chick embryonic skin with that of the prepuce from newborn infants, but also with adult human skin obtained in the course of split skin graft procedures. Moreover, in this series of experiments advantage was taken of the fact that scar tissue of one of the subjects from whom normal skin was obtained could be entered into the plan of the experiment to provide additional data concerning the resistance of fibroblasts to low temperatures.

---

* From the Tissue Culture Laboratory, Medical Branch, The University of Texas, Galveston, and Department of Pathology, USAF School of Aviation Medicine, Randolph Field, Texas. This study was supported by funds provided under Contract AF 41(607)-36 with the USAF School of Aviation Medicine, Randolph Field, Texas. Grateful acknowledgment is made for indispensable aid with tissue culture procedures to Mrs. John Finerty and Mrs. Frank Dunton, to Mr. George Lefeber and Mr. Curtis Hoskins for photomicrographic records and to Mr. McDonald Smith for aid in the preparation of the graphs. Received for publication February 24, 1953.

A successful method for the short term culture of adult human skin has been described by Lewis, Pomerat, and Ezell (1949) which has proven particularly useful in the study of immunological problems associated with homografts (Allgöwer, Blocker, and Engley, 1952), the storage of skin (Allgöwer and Blocker, 1952), the influence of "wound healing agents" (Allgöwer, Pomerat, and Blocker, 1952), and the toxicity of antibiotics (Hu, Livingood, Johnson, and Pomerat, 1953).

Materials and Methods. For the purpose of the present study, tissues were rapidly frozen and thawed, following which they were cultivated and observed according to the methods described in the first report in this series (Pomerat and Lewis, 1953). To assure asepsis, particularly in the management of preputial skin, both 1,000 units of penicillin and 200 gamma of neomycin were incorporated per milliliter of the fluid nutrient.

In contrast to cultures of adult human skin as obtained in split graft procedures, preputial explants from newborn infants contain a considerable proportion of subepithelial tissue which generally yields a relatively large number of spindle cells, presumably fibroblasts, which appear to compete with the epithelial elements in their emigration into the surrounding medium. Mitoses are considerably more numerous in cultures of the skin of the newborn, and cellular emigration is both more rapid and extensive. From some square explants measuring approximately 2 mm. on a side, a halo of radially expanding elements may achieve a diameter of 1 cm. in 7 to 10 days.

Efforts to create conditions which would unmask important variables as described in the first article in this series (Pomerat and Lewis, 1953) included setting up experiments on different days, varying the number of tissue explants to be frozen and when insufficient numbers of preputial explants were available these were distributed across the temperature range under investigation to assure adequacy of sampling.

Results

All essential information is summarized in the protocols and graphs, whereas results of individual experiments are as follows:

A. Preputial Skin of Newborn Infants

1. *Experiments 11–16.* 10 cultures for each condition and cultivated for 12 days. Temperature records in figure 1.

Skin from two babies (C and D) was distributed alternately in experiments across 6 temperatures from —5.5° C. to —29.2° C. so as to help assure adequate sampling. Better outgrowth was obtained from Baby C.

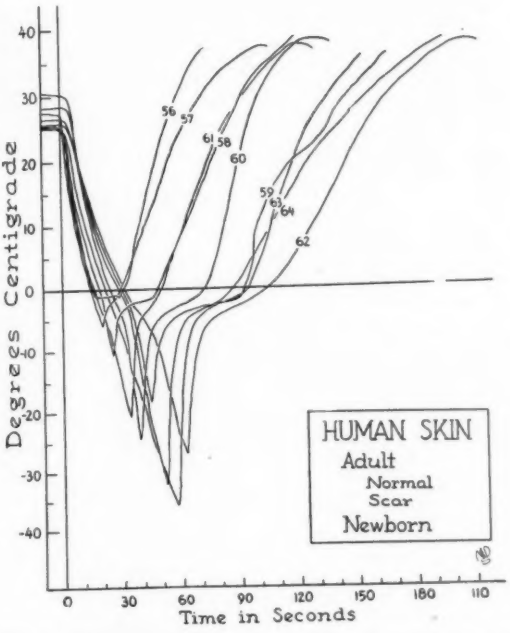


FIG. 1. Thermographic records of freezing and thawing for Experiments 11–16.

Freezing to —11.5° C. in 45 seconds reduced the outgrowth of epithelium moderately while at —15.0° C., which was achieved in 38 seconds, almost total inhibition of epidermal activity was obtained. No epithelium was seen in preparations exposed to —18.2° C. in 35 seconds. While total inhibition was observed in cultures (Baby C—Expt. 15) exposed to —24.7° C.

in 35 seconds, a few spindle cells emigrated in 2 out of 10 cultures after freezing to —29.2° C. in 53 seconds (Baby D—Expt. 16).

2. *Experiments 56–64.* Cultivated for 6 days. Temperature records in figure 2.

Skin from 5 babies (E to I) were again scattered across the temperature range in experiments which extended from

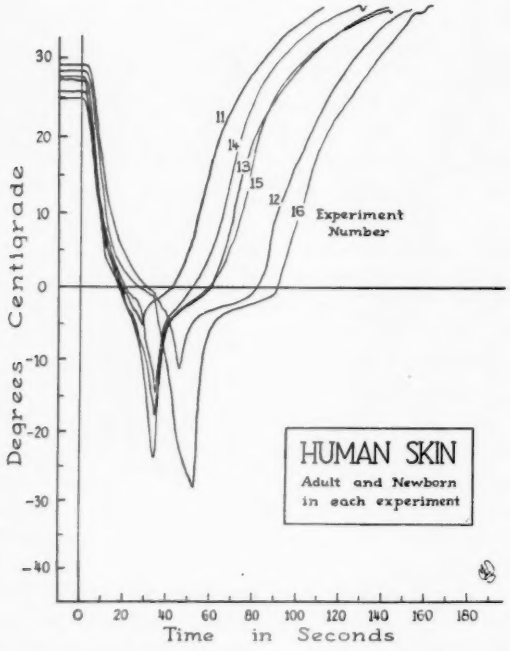


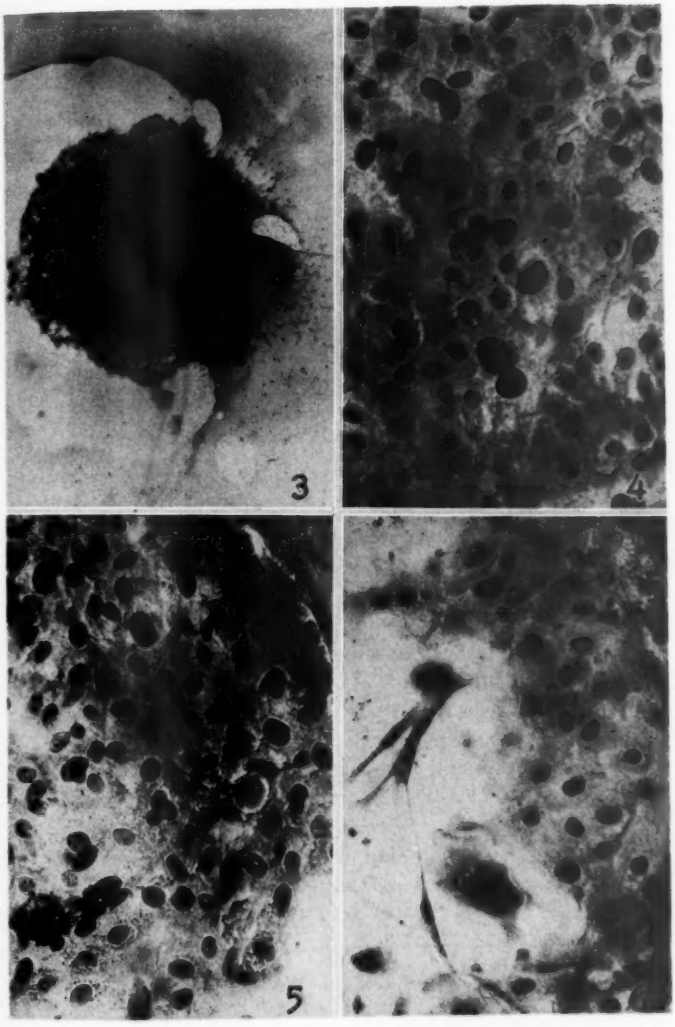
FIG. 2. Thermographic records of freezing and thawing for Experiments 56–64.

FIGS. 3–6. Preputial skin of a newborn infant (Baby I). Cultures for Experiments 56–64 *in vitro* for 6 days.

FIG. 3. Control culture showing both epithelial and spindle cell outgrowth. 12 x.

FIG. 4. Same as figure 3 but at 250 x showing epithelial sheet.

FIGS. 5–6. Representative photomicrographs from the epithelial outgrowth from two explants which had been exposed to freezing to —24.4° C. in 40 seconds. Some spindle cells can be seen in figure 6. 250 x.


3
4
5

—1.4° C. to —35.7° C. Epithelial and spindle cell outgrowth was approximately as dense from Babies E, F, and G, while Babies H and I had somewhat fewer emigrating elements. A typical culture from Baby I is represented in figure 3 at low magnification and at high in figure 4.

Some reduction both in the migration of epithelium and spindle cells resulted from freezing to —10.7° C. in 27 seconds (Expt. 58). While no outgrowth was obtained in 8 out of 20 explants (Baby I) suggesting marked injury after exposure to —24.4° C. in 40 seconds, the epithelium in two cultures appeared relatively uninjured as seen in figures 5 and 6. Some of the spindle cells from this culture are represented in figure 6. No outgrowth was obtained in Experiment 62 (—26.7° C. in 64 seconds).

B. Normal Adult Human Skin

1. *Experiments 11–16.* Cultivated for 12 days. Temperature records in figure 1.

Skin obtained in the course of procedures for split-skin grafts from two adults (A and B) were used in conjunction with the baby skins described as C and D. Patient A yielded a richer outgrowth of cells than Patient B.

Slight injury to both epithelium and spindle fibers was observed in Experiment 12 (—11.5° C. in 45 seconds). While the epithelium was markedly reduced and the spindle cells somewhat less damaged in Experiment 13 (—15.0° C. in 38 seconds), no epithelial outgrowth was obtained after freezing to —18.2° C. in 35 seconds (Adult B—Expt. 14) but a thin outgrowth of spindle cells was present. Similar findings were also obtained from the skin of Patient A after exposure to —24.7° C. in 35 seconds and again from Patient B at an even lower temperature (—29.2° C. in 53 seconds).

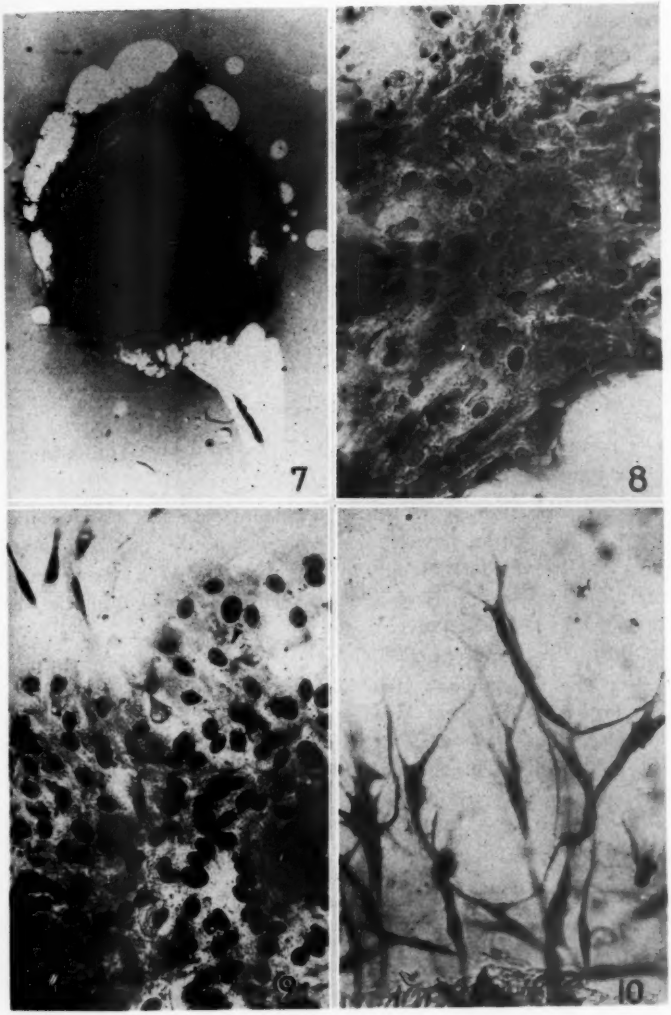
FIGS. 7–10. Normal adult human skin. Cultures from Experiments 56–64 *in vitro* for 6 days.

FIG. 7. Control culture showing both epithelial and spindle cell outgrowth. 12 x.

FIG. 8. Same as figure 7 but at 250 x showing epithelial sheet.

FIG. 9. Epithelial outgrowth from an explant following freezing to —20.5° C. in 35 seconds. 250 x.

FIG. 10. Culture exposed to a temperature of —26.7° C. in 64 seconds. No epithelium was found in the outgrowth but there were a few spindle cells. 250 x.


7
8
9
10

2. *Experiments 56–64.* Cultivated for 6 days. Temperature records in figure 2.

Although two cultures out of 20 controls showed no outgrowth, relatively good epithelial sheets were obtained in the remainder. As is the rule with thin split-skin grafts, few spindle cells were present. A typical culture is represented in figure 7 at low magnification and at high in figure 8.

While no epithelium was obtained in the series exposed to —18.1° C. in 45 seconds (Expt. 59), 4 out of 20 cultures frozen to —20.5° C. in 35 seconds produced epithelium. The best outgrowth from one of these cultures is shown in figure 9. In this series spindle cells but no epithelium were obtained after exposure to a temperature of —26.7° C. in 64 seconds (Fig. 10).

C. Scar Tissue

Experiments 56–64. Cultivated for 6 days. Temperature records in figure 2.

Results obtained with these explants were not too satisfactory except where positive evidence of spindle cell outgrowth was obtained. Many fragments evidently had a very sparse cellular population from which proliferation could not be expected.

Spindle cell outgrowth is shown in figure 11 at low magnification and at high in figure 12 from a typical control culture. It is noteworthy that spindle cell emigration was obtained after exposure to —10.7° C. in 27 seconds (Expt. 58) which resembled those of the controls or cultures of normal adult skin frozen under the same conditions (Fig. 13). While no outgrowth was obtained after freezing to —18.1° C. in 45 seconds, spindle fibers as represented in figure 14 were obtained in one

---

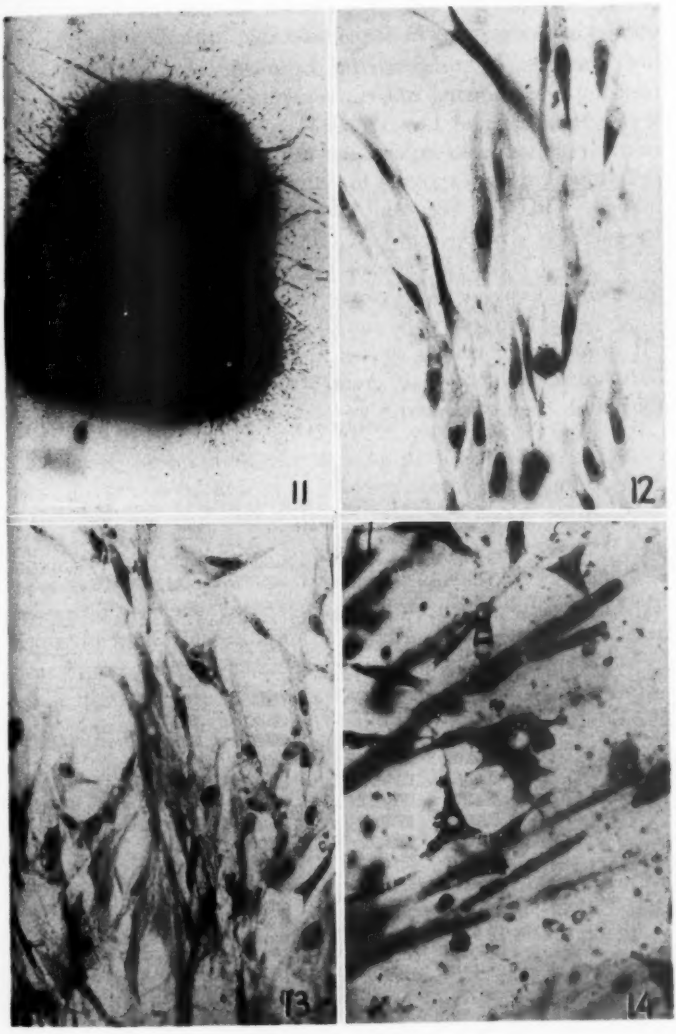
FIGS. 11–14. Human scar tissue. Cultures from Experiments 56–64 *in vitro* for 6 days.

FIG. 11. Control culture showing spindle cell outgrowth. 12 x.

FIG. 12. Same as figure 11 but at 250 x showing pattern of spindle cells in emigration.

FIG. 13. Outgrowth from an explant exposed to —10.7° C. in 27 seconds showing spindle cells. 250 x.

FIG. 14. Damaged cells recorded from one explant which survived after exposure to —18.1° C. in 45 seconds. 250 x.


11
12
13
14

culture exposed to —20.5° C. in 35 seconds. However, these cells exhibited evidences of considerable injury.

Discussion. In summarizing the results obtained with rapid freezing and thawing of skin from 11 and 12 day chick embryos, Pomerat and Lewis (1953) concluded that while slight injury resulted from exposure of as little as —3.4° C., epithelial cells might resist exposure to temperatures as low as approximately —30.0° C. Preputial epithelium persisted after freezing to —24.4° C. while that of adults was not found to emigrate below —20.5° C. In view of the variability of the data, the difference between the survival of newborn and adult human skin does not appear to be significant. Inspection of the graphical summary of results shown in figure 15 suggests the possibility of a slight over-all greater resistance to cold injury by the adult tissue both with respect to epithelium and spindle cells.

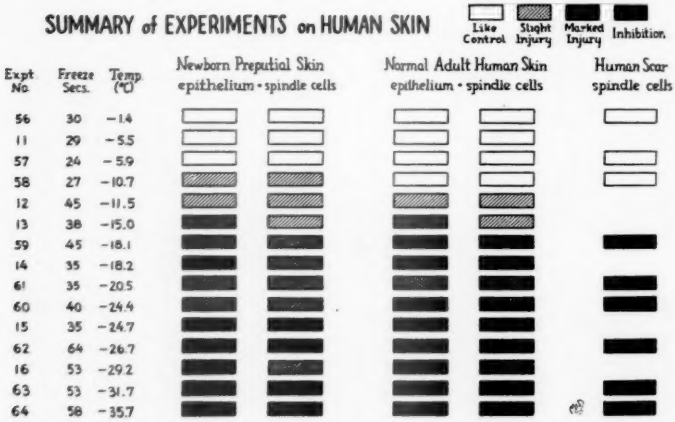


FIG. 15. Composite diagram summarizing results obtained from human skin explants following rapid freezing and thawing.

In contrast to the demonstration that spindle fibers, probably associated with vessels, can emigrate from explants of chick embryonic skin and muscle at temperatures of nearly

—40.0° C., cells of this form appeared to be arrested at approximately —30.0° C. However, it should be remembered that for at least the adult skin graft material very little tissue of mesodermal origin was included in the fragments.

Conclusions

1. Utilizing the technique for cultivating newborn preputial and normal adult skin as well as scar tissue from human sources which was developed by Pomerat and Lewis (1953) for embryonic chick muscle and skin, 680 fragments (including controls) were employed in the study of the effect of freezing and thawing on the viability of epithelial and spindle cells. A range of temperature from —1.4° C. to —35.7° C., generally reached in less than one minute and followed by rapid thawing in a water bath at +42.0° C., was explored.

2. Preputial epithelium was found to survive after exposure to temperatures as low as —24.4° C. achieved in 40 seconds and adult human epidermis produced emigrating cells following freezing to —20.5° C. in 35 seconds.

3. Spindle cells survived exposure to —29.2° C. produced within 53 seconds from both preputial and adult skin. Cells of this form emigrated from chick embryonic muscle and skin after exposure to temperatures approximately 10 degrees lower. While the problem was complicated by the relatively large proportion of amorphous inert material, fragments of human scar tissue showed outwandering spindle cells after freezing at —20.5° C. which was achieved in 35 seconds.

REFERENCES

Allgöwer, M., and T. G. Blocker, Jr.: Viability of skin in relation to various methods of storage. *Texas Rep. Biol. and Med.*, 10: 3–21, 1952.

Allgöwer, M., T. G. Blocker, Jr., and B. W. D. Engley: Some immunological aspects of auto- and homografts in rabbits, tested by *in vivo* and *in vitro* techniques. *Plast. and Recon. Surg.*, 9: 1–21, 1952.

Allgöwer, M., C. M. Pomerat, and T. G. Blocker, Jr.: Influence of normal serum, its derivatives, and of "wound healing agents" on human epidermis *in vitro*. *Annals of Surg.*, 135: 923–937, 1952.

Hu, Funan, C. S. Livingood, Patricia Johnson, and C. M. Pomerat: Tissue Culture Studies on Human Skin. IV. The comparative toxic effects of antibiotics on tissue culture explants of human skin and embryonic chick spleen. *J. Inv. Derm.* (in press), 1953.

Lewis, S. R., C. M. Pomerat, and D. Ezell: Human epidermal cells observed in tissue culture with phase-contrast microscopy. *Anat. Rec.*, 104: 487–504, 1949.

Pomerat, C. M., and R. B. Lewis: Final contract report (unpublished), 1953.

Protocols for Experiments on Newborn and Adult Skin

	NEWBORN PREPUTIAL SKIN	ADULT NORMAL SKIN
CONTROL—12 day	Baby C	Adult A
For Experiments 11–16	Considerable liquefaction with many spindle cells but 9/10 have epithelium. No outgrowth in 1/10.	Good outgrowth of epithelium in 8/10. Numerous spindle cells. No outgrowth in 1/10.
	Baby D	Adult B
	Less outgrowth than from explants of Baby C. Epithelium in 4/10 and spindle cells only in 6/10.	Somewhat less outgrowth than Adult A.
EXPERIMENT 11	Baby C	Adult A
—5.5°C. in 29 seconds	Like the control.	Like the control.
EXPERIMENT 12	Baby D	Adult B
—11.5°C. in 45 seconds	Outgrowth a little less than the control. Epithelium relatively more damaged than the spindle cells.	Outgrowth somewhat less than the control.
EXPERIMENT 13	Baby C	Adult A
—15.0°C. in 38 seconds	Spindle cells less numerous and more scattered than in control. Out of 10 cultures there is good epithelium in one and poor in another.	Spindle cells slightly reduced. Epithelium only in 1/10. No outgrowth in 5/10.
EXPERIMENT 14	Baby D	Adult B
—18.2°C. in 35 seconds	Reduced spindle cell outgrowth. No epithelium.	Spindle cells reduced. No epithelium.
EXPERIMENT 15	Baby C	Adult A
—24.7°C. in 35 seconds	No outgrowth.	A few spindle cells in 2/10. No outgrowth in 8/10.
EXPERIMENT 16	Baby D	Adult B
—29.2°C. in 53 seconds	Very few spindle cells seen in 2/10.	Outgrowth of a few spindle cells in 4/10.

Protocols for Experiments on Newborn Preputial Skin

CONTROLS—6 day For Experiments 56–64	
Baby E Epithelium in 17/17	EXPERIMENT 56—Baby E —1.4°C. in 30 seconds. Epithelium in 9/15 like control. Spindle cells only in 6/15. No outgrowth in 1/15.
Baby F Epithelium in 14/14	EXPERIMENT 57—Baby F —5.9°C. in 24 seconds. Epithelium in 15/17. Spindle cells only in 1/17.
Baby G Epithelium in 13/13	EXPERIMENT 58—Baby G —10.7°C. in 27 seconds. Epithelium in 6/20. Spindle cells only in 7/20. No outgrowth in 7/20.
Baby H Epithelium in 11/13 Spindle cells only in 1/13 No outgrowth in 1/13	EXPERIMENT 59—Baby H —18.1°C. in 45 seconds. Few cells but epithelium in 4/18. Spindle cells only in 2/18 and no outgrowth in 12/18.
Baby I Epithelium in 12/15 Spindle cells only in 1/15 No outgrowth in 2/15	EXPERIMENT 60—Baby I —24.4°C. in 40 seconds. Poor outgrowth but epithelium in 11/20. Spindle cells only in 1/20 and no outgrowth in 8/20.
	EXPERIMENT 61—Baby E —20.5°C. in 35 seconds. Poor outgrowth but epithelium in 4/18. Spindle cells only in 2/18 and no outgrowth in 12/18.
	EXPERIMENT 62—Baby F —26.7°C. in 64 seconds. No outgrowth from 18 explants.
	EXPERIMENT 63—Baby G —31.7°C. in 53 seconds. No outgrowth from 13 explants.
	EXPERIMENT 64—Baby I —35.7°C. in 58 seconds. No outgrowth.

Protocols for Experiments on Adult Human Tissue

	ADULT NORMAL SKIN	SCAR TISSUE
CONTROL—6 day For Experiments 56–64	Epithelium in 17/20 usually with scant spindle outgrowth. No cell emigration in 2/20.	Explants made up of wavy fibers and amorphous masses. Spindle cells from 6/20. No outgrowth in the remainder.
EXPERIMENT 56 —1.4°C. in 30 seconds	Like control. Epithelium in 17/19.	Spindle cells in 16/18.
EXPERIMENT 57 —5.9°C. in 24 seconds	Outgrowth from all explants. Epithelium in 8/20.	Spindle cells in 9/17.
EXPERIMENT 58 —10.7°C. in 27 seconds	Epithelium in 15/19. Spindle cells only in one. No outgrowth in 3/19.	Spindle cells in 15/20.
EXPERIMENT 59 —18.1°C. in 45 seconds	No epithelium. Spindle cells in 3/20. No outgrowth in the remainder.	No outgrowth.
EXPERIMENT 60 —24.4°C. in 40 seconds	No epithelium. A few spindle cells in 2/20.	No outgrowth.
EXPERIMENT 61 —20.5°C. in 35 seconds	Epithelium in 4/20. Spindle cells only in 6/20. No outgrowth in the remainder.	Spindle cells in 1/20. No outgrowth in the remainder.
EXPERIMENT 62 —26.7°C. in 64 seconds	No outgrowth.	No outgrowth.
EXPERIMENT 63 —31.7°C. in 53 seconds	No outgrowth.	No outgrowth.
EXPERIMENT 64 —35.7°C. in 58 seconds	No outgrowth.	No outgrowth.

COMPARISON BY ELECTRON MICROSCOPY OF THE NTAYA AND ZIKA VIRUSES*

REGINALD L. REAGAN AND A. L. BRUECKNER

The viruses employed in these studies were the Ntaya virus and Zika virus furnished by the American Type Culture Collection in Washington, D.C. These 2 viruses were obtained by the above institution from Dr. K. C. Smithburn (Smithburn and Haddow, 1951). The Ntaya virus and Zika virus were encountered by these workers in field investigations to discover the insects responsible for the transmission of yellow fever in extra-human cycles of natural infection in Uganda Protectorate, British East Africa. Mosquitoes were captured in selected localities and classified. Suspensions of these mosquitoes were inoculated into susceptible laboratory animals (mice and rhesus monkeys) to test for the presence of virus.

Materials and Methods. Twenty Swiss albino mice (3-weeks-old) were inoculated intracerebrally with 0.03 cc. of lyophilized mouse brain (Ntaya virus strain of the 16th mouse passage) and 20 Swiss albino mice (3-weeks-old) were inoculated intracerebrally with 0.03 cc. of lyophilized mouse brain (Zika virus strain of the 146th mouse passage). The mice showed nervous sypmtoms of mild tremors, convulsions, and paralysis after an incubation period of 5 to 7 days. When symptoms appeared, the mice from each group were sacrificed separately, and the brains and cords were removed aseptically. Twenty mice were injected intracerebrally with 10 per cent suspensions of normal mouse brains. No symptoms were noted in these normal controls. These 20 mice were sacrificed on the 6th day post inoculation. The brains and cords were removed aseptically as above. Pools of Ntaya and Zika infected mouse brains and of normal mouse brains and cords were ground with alundum, and diluted to 10 per cent suspensions with physiological saline. The 3 suspensions were then centrifuged for 5 minutes in an angle centrifuge at 2,000 r.p.m. The superna-

* From the Live Stock Sanitary Service Laboratory, Maryland State Board of Agriculture, University of Maryland, College Park, Maryland.
Received for publication February 16, 1953.

tants were removed from all 3 specimens and filtered through type ST size L3 Seitz filters. The filtrates were then subjected to centrifugation under refrigeration for 1 hour at 44,770 r.p.m. The temperature of the refrigerated outer jacket stayed constant at —10° C. during the centrifugation. After the 1-hour centrifugation period, the supernatants from all 3 specimens were discarded, and the sediment from each specimen was resuspended in 0.8 cc. of distilled water. Several drops of each suspension were placed on several parlodion film supports which had been prepared 72 hours previously. After all excess fluid had been removed with small capillary pipettes, the films were dried and shadowed with chromium (Williams and Wyckoff, 1945) at art tangent 2/10 and examined under the R.C.A. electron miscroscope, type E.M.U. The balance of the material of each suspension was injected intracerebrally into each of six 3-week-old Swiss almino mice.

The mice injected with the concentrated infected material of the Ntaya virus strain showed nervous symptoms after a period of 7 days. The mice injected with the concentrated material of the Zika virus strain showed nervous symptoms after a period of 8 days. Mice from each group were sacrificed and the brains were removed aseptically, pooled, ground, and diluted to a 10 per cent suspension. The suspension from the Ntaya infected mice was injected intracerebrally into 4 unvaccinated mice and into 4 mice which previously had been immunized intramuscularly with the original Ntaya virus. After 6 days the unvaccinated mice showed nervous symptoms; while the immunized group showed no nervous symptoms. This confirmed the concentrated virus to be Ntaya virus. The suspension from the Zika infected mice was also injected intracerebrally into 4 unvaccinated mice and into 4 mice which previously had been immunized intramuscularly with the original Zika virus. After 8 days the unvaccinated mice showed nervous symptoms; while the immunized group showed no nervous symptoms. This confirmed the concentrated virus to be Zika virus. The mice injected intracerebrally with the concentrated suspensions of normal mouse brains and cords appeared normal and were discarded after a 21-day observation period.

Upon electron microscopic examination of the concentrated suspension of the brains and cords of the normal mice, no virus-like particles could be seen. These controls were screened carefully. Upon examination of the concentrated suspensions from the infected brains and cords of the Ntaya virus, uniform virus-like particles were demonstrated as shown in figure I.

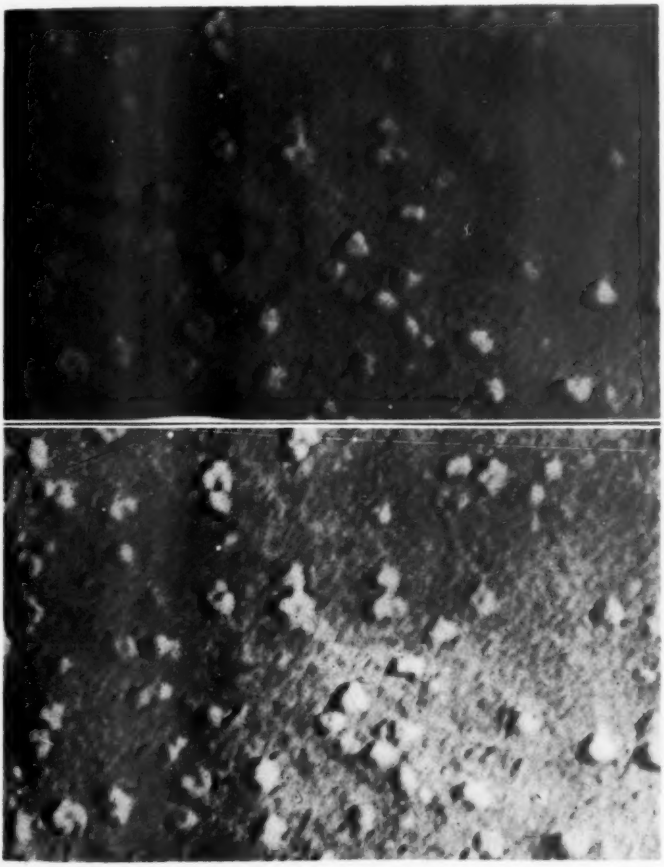


FIG. I. Ntaya virus shadowed with chromium at arc tangent 2/10, X72,500.

These bodies measure 80 mμ by direct measurement and are cube shaped with irregular contour and resemble the West Nile virus described by Reagan *et al.* (Reagan and Brueckner, 1951). Upon examination of the concentrated suspensions from the infected brains and cords of the Zika virus, uniform virus-like particles were demonstrated as shown in figure II. These

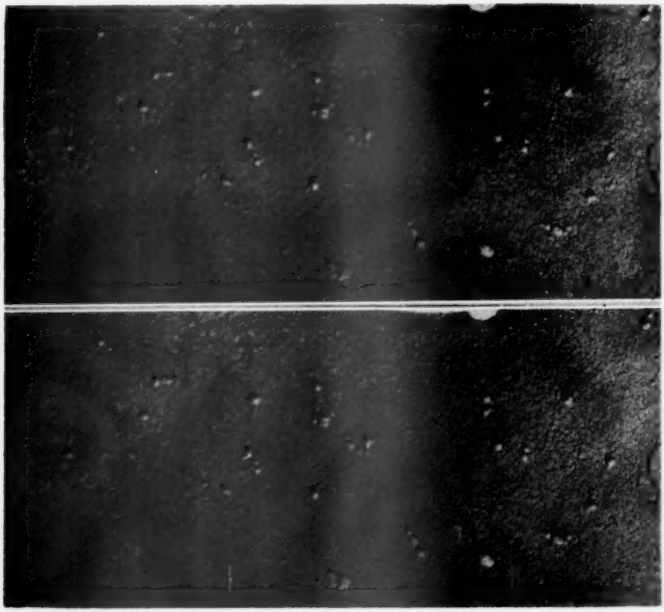


FIG. II. Zira virus shadowed with chromium at arc tangent 2/10, X40,000.

bodies measure 40 mμ by direct measurement and are spherical in shape.

Summary

Studies by electron microscope of brains and spinal cords of mice infected with Ntaya fever virus show the virus to be cube shaped with a diameter of 80 mμ in comparison to the Zika virus which is spherical with a diameter of 40 mμ. These

bodies could not be demonstrated in concentrated normal mice brains and spinal cords subjected to the same procedure of concentration and examination. The bodies of Ntaya virus resemble the West Nile virus bodies. The virus in the concentrated infectious material from Ntaya was confirmed to be Ntaya virus and the concentrated infectious material from Zika was also confirmed to be Zika virus.

REFERENCES

Smithburn, K. C., and A. J. Haddow: Ntaya. A hitherto unknown agent isolated from mosquitoes collected in Uganda. *Proc. Soc. Exp. Biol. and Med.*, 77: 130–133, 1951.

Williams, R. C., and R. W. G. Wykoff: Applications of metallic shadow-casting to microscopy. *J. Applied Physics*, 17: 23–33, 1946.

Reagan, Reginald L., and A. L. Brueckner: Morphological observations by electron microscopy of West Nile virus after propagation in the Swiss mouse. *Proc. Soc. Exp. Biol. and Med.*, 78: 630–63, 1951.

PAPER ELECTROPHORESIS OF HEMOGLOBIN AS A PRACTICAL METHOD OF DIFFERENTIATING VARIOUS TYPES OF SICKLE CELL DISEASE AND OF HEMOGLOBIN "C" TRAIT*

ROSE G. SCHNEIDER

During the course of experiments on the immunological characteristics of erythrocytes from patients with sickle cell anemia and sickle cell trait (Schneider and Levin, 1950; Schneider, unpublished experiments), it became pertinent to correlate these immunological characteristics with the types of hemoglobin in the erythrocytes.

The erythrocytes of the sickle cell anemia patient have been found by Pauling *et al.* (1949) to contain almost 100 per cent of a type of hemoglobin which has a slightly higher isoelectric point than does normal hemoglobin. Erythrocytes of individuals with sickle cell trait contain a mixture of the normal and sickle cell anemia types. Still another clinically significant type of hemoglobin which migrates as an even more positive ion than that of the sickle cell anemia type has been described by Itano and Neel (1950) and by Kaplan, Zuelzer, and Neel (1951) and designated hemoglobin "C" (Itano, 1951). These differences have been established by electrophoresis. However, this procedure is impractical for general use; therefore, the differentiation of these types of hemoglobin was attempted by means of the simpler, less expensive method of paper electrophoresis (Durram, 1950; Flynn, 1951; Kunkel and Tiselius, 1951).

Materials and Methods. The original experiments were made with the Durrum aparatus (1950); however, early in the course of our work an apparatus was developed which gave better and more rapid separations. It was assembled from inexpensive and readily available materials. As shown in figure I, this apparatus utilizes a wide strip of filter paper draped over glass rods, with the ends dipping into two buffer chambers, each connected by wicks with its own electrode chamber, and the whole

* From the Tissue Culture and Tissue Metabolism Research Laboratories, The University of Texas Medical Branch, Galveston. Aided by U.S.P.H. Grant No. RG-3058(R). Received for publication March 10, 1953.

covered to prevent drying. Each chamber measures 5 x 19.5 x 7.5 cm. In practice a rectangular plastic dish is divided lengthwise in the middle with a plastic partition to form each paper-chamber electrode-chamber system. Glass U tubes are filled with buffer and placed between the chambers to equalize the fluid levels. They are removed before starting the experiment. A similar siphoning device between the electrode chambers remains in place during the run so that changes of fluid level due to electrolysis of water are equalized. The chambers, with the paper arranged as shown, are placed on a plastic tray with a tightly-fitting cover. Holes bored in both sides of this cover permit the insertion of graphite electrodes. Wicks carry the current from the electrode to the paper chambers with minimal pH changes in the latter.

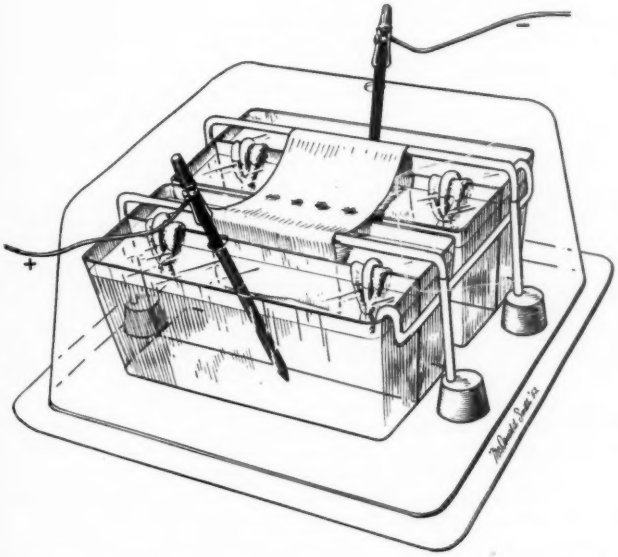


FIG. I. Paper Electrophoresis Apparatus—The paper and electrode chambers are connected by means of wicks (these are tied to U tubes merely to keep them rigid. The buffer-filled U tube between the electrode chambers serves to equalize changes in the fluid levels due to electrolysis of water. The paper is arranged with a slight depression in the middle, with an incline leading up to each glass rod over which it is draped with the ends hanging in the buffer bath.

Munktell 20 paper (Kunkel and Tiselius, 1951) is arranged with a slight depression in the middle, from which there is a gradual incline on either side leading to a glass rod over which the paper is draped with the

ends dipping into the inner buffer chambers. The slight incline has been found to improve separation of proteins.[1] The depression facilitates placement of the hemoglobin drop so that it does not run down the paper. Moreover, when two types of hemoglobin are expected to migrate in opposite directions, this arrangement with the slight incline on either side is of value. A few experiments with Whatman No. 1 paper indicated that this type of paper may also be satisfactory.

The whole apparatus is placed in a refrigerator at 6–10° C., and the door of the refrigerator is closed on the wires leading to the power source. A few experiments made at room temperature were unsatisfactory, probably because room temperatures were high. The original power source consisted of 7 to 9 forty-five volt B batteries connected in series. These were satisfactory for paper strips up to 8 cm. in width. At present a variable voltage regulated power supply is used. This permits the use of wider strips, up to the limit of the space available (about 14 cm.). Anomalous results are sometimes observed on the edges of the strip so that it is advisable to leave ½ to 1 cm. free along each edge. A current of about 7–20 milliamperes and 200–400 volts (0.5 to 1.3 ma. per cm. width and 8–16 V. per cm. length) is applied for 2 to 8 hours. Voltage readings remain constant during the run, but there is a gradual rise in current; if the latter becomes too high for a proonged time (above 20 ma.) results are unsatisfactory, possibly because the heat of the current produces denaturation of the hemoglobin. The introduction of a resistor into the system permits the maintenance of constant amperage.

Hemoglobin is prepared either according to the method of Drabkin (1946), or simply by hemolyzing the washed packed erythrocytes overnight with ½ volume of distilled water and ¼ volume of ether. The hemolysate is clarified by centrifugation at approximately 3,000 r.p.m. for 1 hour at 10° C. Unless otherwise noted, patients have had no transfusions for a period of three months before blood samples for analysis are drawn. Hemoglobin concentrations are determined spectrophotometrically with a Beckman DU spectrophotometer. The hemoglobin solutions are diluted to from 2 to 5 per cent with buffer. Five per cent concentrations give more intense color but migration is less than at 2 per cent. Phosphate, cacodylate, and veronal buffers at 0.05 ionic strength have been used. A few experiments have been made with borate and glycine buffers, but with these the results were not dependable. Normal blood samples are obtained from the blood bank; those from Negro donors are tested for the presence of sickling. All the sickle cell anemia patients have given unequivocal clinical evidence of this disease[2] and all but one have reacted positively with anti-sickle cell anemia serum (Schneider and Levin, 1950). Tiselius

[1] We are indebted to Dr. Charles T. Knorp, Dept. of Bacteriology, University of Michigan Medical School, Ann Arbor, Michigan, for this and other suggestions.

[2] The clinical studies were made under the direction of Dr. W. C. Levin, Director, Hematology Research Laboratory, The University of Texas Medical Branch, Galveston.

electrophoretic analyses of some of the corresponding carbonmonoxyhemoglobin or ferrohemoglobin samples were made with the Perkin Elmer Tiselius Electrophoresis Apparatus, Model 38 (Pauling, Itano, Singer, and Wells, 1949). Some paper electrophoresis analyses have been made with ferrohemoglobin and carbonmonoxyhemoglobin; however, at slightly alkaline pH levels, oxyhemoglobin is satisfactory. Most experiments were made with veronal buffer at pH 8.6 to 9.0; since the most dependable results were obtained with these. The pH values are determined by means of the Beckman Model G pH meter.

Technique. About 500 ml. of cold buffer is placed in each of the four chambers. Buffer-filled U tubes are inserted between the chambers to equalize the levels. Munktell 20 paper, 25 cm. long and 8–14 cm. wide, is draped as shown in figure I with the ends dipping into the buffer baths. The paper becomes moistened by capillary action during a 15-minute period of equilibration during which time the fluid levels are equalized and the apparatus is cooled and stabilized. One-hundredth ml. of 2 to 5 per cent hemoglobin solution is then placed on each of the pencil lines in the center of the paper. Current is applied to the apparatus in the refrigerator. At the conclusion of the run, usually 2 to 8 hours, the paper is carefully lifted off the glass rods and placed immediately on a flat metal or glass plate in an oven at 80–100° C. The paper is smoothed down over the hot plate to prevent movement of the hemoglobin by capillary action due to uneven drying. For most purposes staining is not essential, since the hemoglobin color itself is clearly visible, especially with 5 per cent solutions. When staining is desired, a slight modification of the technique of Durram (1950) is used; the papers are placed in a 95 per cent ethanol solution containing 0.1 gm./per cent brom phenol blue and 10 gm./per cent mercuric chloride. After staining for 15 minutes, they are washed in running tap water for 10 minutes.

For consistently good results, a number of minor precautions must be taken. The amount of buffer in all chambers must be fairly large and the levels must be equalized. The hemoglobin should follow a course against gravity, and must not be denatured by heat or drying during the run. Electrical conditions over the width of the strip must be uniform. To determine this, a few trial runs with several aliquots of the same hemoglobin solution are advisable. The blood samples for making the hemoglobin and the hemoglobin solutions themselves must be relatively fresh. Hemoglobin solutions kept for several weeks in the refrigerator give marked trailing effects. Finally, care must be taken in the drying process so that the hemoglobin spots remain in their original position.

Results

In 0.05 ionic strength phosphate, cacodylate, or veronal buffers of between pH 6.5 and 8.6, all the types of hemoglobin migrate towards the cathode, with the sickle cell type migrat-

ing farther than the normal; at the lower pH values there is considerable trailing of the protein. In veronal buffer at pH 8.6, the sickle cell anemia hemoglobin moves towards the cathode, while the normal hemoglobin remains stationary or moves slightly to the anode. The hemoglobin of the sickle cell trait patient or artificial mixtures of the sickle cell anemia and the normal hemoglobin solutions are clearly distinguished from either the normal or the sickle cell anemia hemoglobin alone; for part of the hemoglobin of the trait or the mixture remains stationary while another part moves towards the cathode. The resulting band is wider than that of either the sickle cell anemia or the normal homoglobin alone and overlaps the two. Wells and Itano (1951) have shown that there is 24 to 45 per cent of sickle cell anemia hemoglobin in the erythrocytes of the sickle cell trait patient. In known mixtures of normal and sickle cell anemia hemoglobins, 24 to 45 per cent of the latter may easily be distinguished from the sickle cell anemia hemoglobin alone, as illustrated in figures III-A and III-B. With 15–20 ma. current and 200–400 V. (1 to 1½ ma./cm. width and 8–16 V./cm. length), the sickle cell anemia hemoglobin has migrated completely off the starting line in two to three hours, a characteristic which differentiates it clearly from the sickle cell trait hemoglobin, a portion of which remains at the starting boundary. In a run of 8 hours, the normal hemoglobin still remains stationary while the sickle cell anemia hemoglobin has migrated a considerable distance towards the cathode. The mixtures of the two, or the sickle cell trait hemoglobin, are clearly in an intermediate position. In about 6 hours, two distinct bands may often be seen (Fig. IV-B). At pH 9.0, the sickle cell anemia hemoglobin remains at the line, while the normal hemoglobin migrates slightly to the anode and hemoglobin "C," slightly to the cathode; the sickle cell trait hemoglobin or a mixture of normal and sickle cell anemia hemoglobins occupy an intermediate position between the normal or sickle cell anemia hemoglobins alone. In buffers of pH 8.6 to 9.0, hemoglobin "C" migrates farther to the cathode than does the sickling hemoglobin. In glycine buffer at pH 9.7, both the normal and sickling types of hemoglobin migrate to the anode while hemoglobin "C" remains stationary or migrates

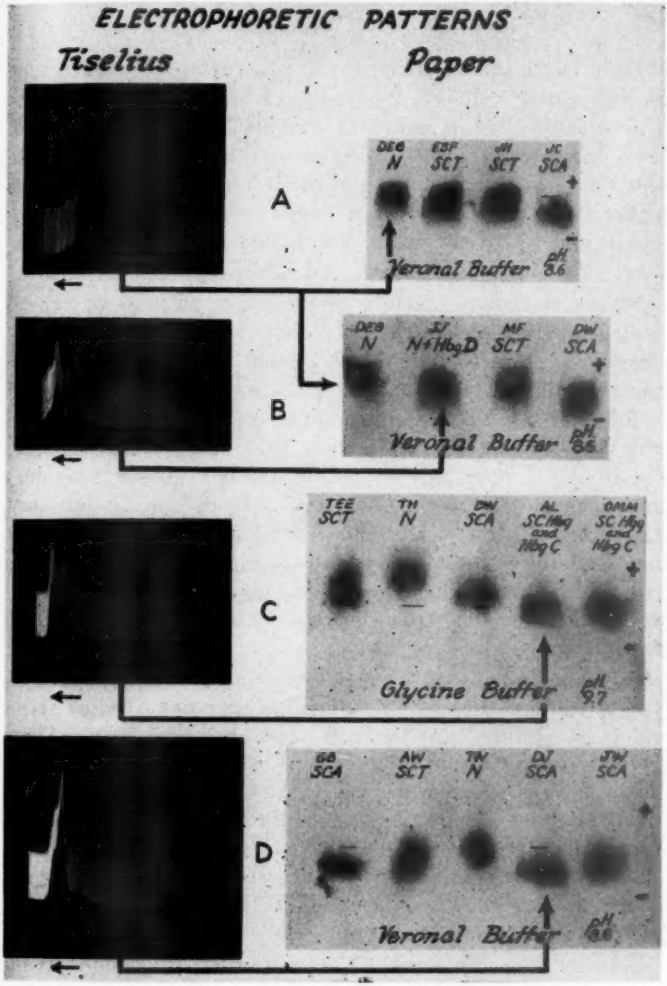


FIG. II. Tiselius Patterns—Ascending limb of carbonmonoxyhemoglobin or ferrohemoglobin in cacodylate buffer pH 6.5. Paper Patterns—A.) 0.01 cc. 5 per cent hemoglobin, 2½ hours, current source: nine 45 V. B. batteries in series (unstained); B.) 0.01 cc. 4 per cent hemoglobin solutions; 2¼ hours, current source: seven 45 V. B. batteries in series (unstained); C.) 0.01 cc. 3 per cent hemoglobin solutions, 3 hours, 250 V. 13 to 20 ma. (unstained); D.) 0.01 cc. 3 per cent hemoglobin solutions, 3½ hours, 250 V. 7 to 15 ma. (unstained); SCA—sickle cell anemia; SCT— sickle cell trait; N—normal.

slightly to the cathode (Fig. II-C). The pH at which each type of hemoglobin remains stationary varies somewhat with the type of buffer and its ionic strength; with the concentration of the hemoglobin; and/or with different current conditions. These apparent isoelectric points are about two or more pH units higher than the true isoelectric points for carbon monoxyhemoglobin and ferrohemoglobin determined in the Tiselius apparatus (Pauling, Itano, Singer, and Wells, 1949).

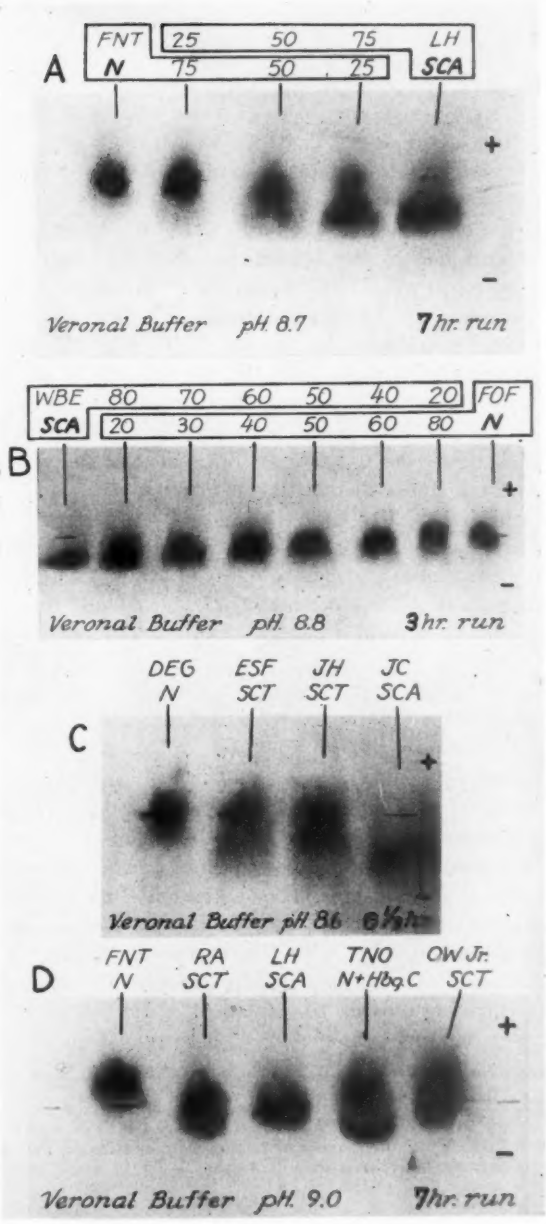
Figure II shows a picture of the paper patterns of typical hemoglobin samples with the corresponding Tiselius electrophoretic pattern. In figure II-B, patient JJ is noteworthy: she has two siblings with sickle cell anemia (one of these is DJ in figure II-D). Both parents and two other siblings have sickle cell trait. Repeated sickling tests on JJ have been negative; but in a 2½ hour run on paper, her hemoglobin resembles that of an individual with sickle cell trait. In the Tiselius electrophoresis apparatus, JJ's hemoglobin separates into two peaks corresponding to those described by Itano (1951) in individuals with normal hemoglobin and hemoglobin "D." This hemoglobin "D" has the same electrophoretic mobility as sickling hemoglobin, but does not produce sickling.

The combination of normal hemoglobin and still another type called hemoglobin "C", which has been described by Itano and Neel (1950) may be readily identified on paper. Since erythrocytes with the combination of these two types of hemoglobin have a shortened survival time in transfusion experiments (Kaplan, Zuelzer, and Neel, 1951) a test to differentiate them from normal erythrocytes may be of interest. Several examples of the combination of normal hemoglobin and hemoglobin "C" are shown in figure IV-B, where after a 6½ hour run, two distinct zones have appeared.

Two patients with sickle cell trait had hemoglobin giving patterns different than those usually seen in sickle cell trait

---

FIG. III. A.) Mixtures of SCA and normal hemoglobin in different proportions 0.01 cc. of 3 per cent hemoglobin, 300 V. 13–20 ma. (stained); B.) Mixtures of SCA and normal hemoglobin in different proportions 0.01 cc. of 5 per cent hemoglobin solutions, 350 V. 15–20 ma. (unstained); C.) Same as figure II-A except for time of run (unstained); D.) 0.01 cc. of 3 per cent hemoglobin solutions, 7 hours, 300 V. 13–20 ma. (stained).


A
FNT
N
25
75
50
50
75
25
LH
SCA
+
−
Veronal Buffer pH. 8.7
7hr. run
B
WBE
SCA
80
20
70
30
60
40
50
50
40
60
20
80
FOF
N
+
−
Veronal Buffer pH. 8.8
3hr. run
C
DEG
N
ESF
SCT
JH
SCT
JC
SCA
+
Veronal Buffer pH 8.6
D
FNT
N
RA
SCT
LH
SCA
TNO
N+Hbg C
OW Jr.
SCT
+
−
Veronal Buffer pH. 9.0
7hr. run

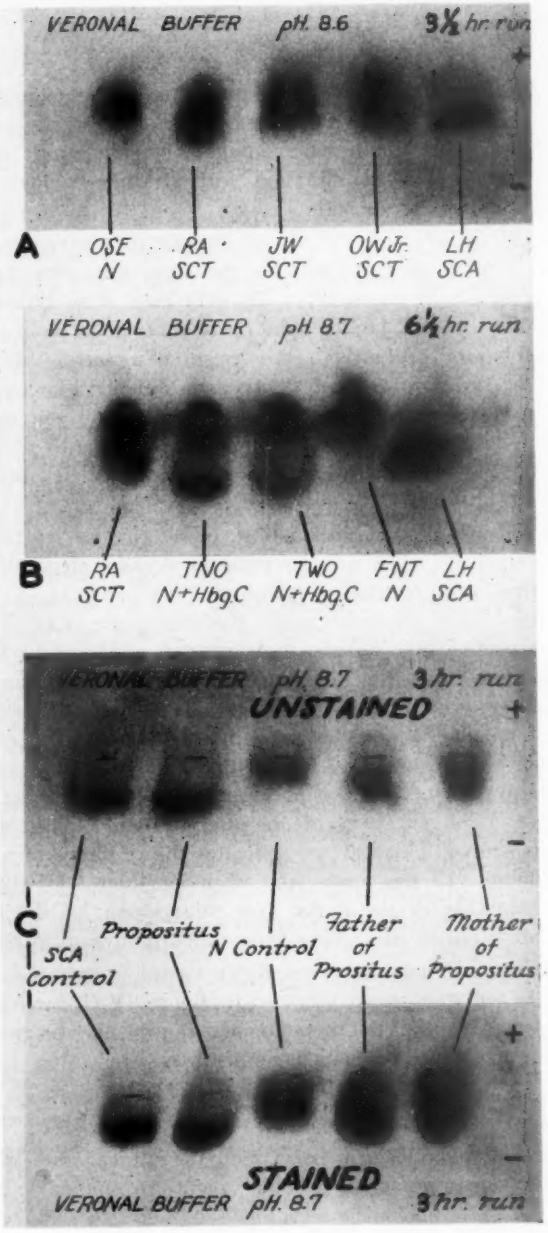
patients. Both of these patients were interesting clinically because of hepatosplenomegaly, red blood counts of 4 or more million and an increased reticulocyte count. In paper electrophoresis, at pH 8.6 for 3 hours, the hemoglobin of both of these individuals migrated farther towards the cathode than did the hemoglobin of the sickle cell anemia patients. Figure II-C shows the pattern made by the hemoglobin of these two patients in glycine buffer at pH 9.7 in comparison with one normal hemoglobin sample, one from a patient with sickle cell trait and one with sickle cell anemia. At this pH the normal hemoglobin has moved towards the anode, the hemoglobin of the patient with sickle cell anemia is concentrated slightly to the anode side of the starting line and that of the sickle cell trait patient is situated in an overlapping position between these two. The hemoglobin of the two patients in question (AL and OMM) is concentrated slightly towards the cathode side of the starting line. These patients are believed to have a combination of sickling hemoglobin and hemoglobin "C." The Tiselius electrophoresis pattern of one of them (AL) bears out this conclusion, as do the clinical findings (Kaplan, Zuelzer, and Neel, 1951).

We have to date tested hemoglobin samples from 22 patients with sickle cell anemia, from 29 with sickle cell trait and from 65 normal subjects. Three subjects with hemoglobin "C" in combination with normal hemoglobin have been found.

In seven of the patients with clinical sickle cell anemia there is a demonstrable admixture of hemoglobin "C" with the sickling hemoglobin. Such an admixture may be seen in patient JC, figures II-A and III-C. This patient is a 65-year-old man with typical clinical sickle cell anemia, a disease generally considered incompatible with this degree of longevity. In repeated experiments on paper electrophoresis, his hemoglobin migrates farther towards the cathode (at pH 8.6 to 9.0) than does known sickling hemoglobin. At least two of the 29 patients

---

FIG. IV. A.) 0.01 cc. of 3 per cent hemoglobin solution 300 V. 14–17 ma. (unstained); B.) 0.01 cc. of 3 per cent hemoglobin 300 V. 13–23 ma. (stained); C.) 0.01 cc. of 3 per cent hemoglobin solution of patient W.C. and of his parents, together with a SCA control (WH) and a normal control (FNT).


VERONAL BUFFER pH. 8.6 3½ hr. run
+
−
A
OSE N
RA SCT
JW SCT
OW Jr. SCT
LH SCA
VERONAL BUFFER pH. 8.7 6½ hr. run
B
RA SCT
TNO N+Hbg.C
TWO N+Hbg.C
FNT N
LH SCA
VERONAL BUFFER pH. 8.7 3 hr. run
UNSTAINED
+
−
C
SCA Control
Propositus
N Control
Father of Prositus
Mother of Propositus
+
−
STAINED
VERONAL BUFFER pH. 8.7 3 hr. run

with sickle cell trait have large quantities of hemoglobin "C" (AL and OMM in Fig. II-C). Additional studies are required to determine the smallest percentages of hemoglobin "C" that can be detected in combination with sickling hemoglobin or normal hemoglobin or both.

It has not been possible to standardize conditions from one run to another so that precisely the same migration patterns can be obtained repeatedly. To overcome this difficulty, a sample of normal hemoglobin or one of sickle cell anemia hemoglobin or preferably both must be included with each group of unknowns. With these as a reference point, it is usually easy to differentiate between the sickle cell anemia, sickle cell trait, and normal hemoglobins in a run of 2 to 3 hours. When the presence of hemoglobin "C" is suspected, runs of 6 to 8 hours are advisable.

While this method does not give the precise quantitative measurements that are possible with the classical electrophoresis apparatus, an approximate estimate of the percentages of normal and sickling hemoglobin in a given sample may be obtained by comparing this sample with several mixtures of known composition that are analyzed simultaneously. Using this method, we have found it possible to detect as little as 10 per cent of normal hemoglobin in combination with 90 per cent of sickle cell hemoglobin. In figure II-D, hemoglobin samples of several patients with clinical sickle cell anemia are compared. A Tiselius pattern of one of these hemoglobin samples (DJ) is shown. This pattern indicates the presence of normal hemoglobin (12 per cent) and of hemoglobin "C" (26 per cent). Staining of the paper and enlargement of the photograph may aid in measuring these amounts quantitatively.

This method may be particularly useful in genetic studies of sickle cell disease as indicated in figure IV-C, since hemoglobin samples of all the members of a family may be analyzed simultaneously for comparative purposes.

Discussion

There long has been a need, both for clinical and research purposes, for a practical, reliable diagnostic method to differ-

entiate patients with sickle cell anemia from those with an unrelated anemia and sickle cell trait. The electrophoretic analysis of the hemoglobin of these patients by means of a Tiselius apparatus (Pauling, Itano, Singer, and Wells, 1949) undoubtedly offers an exact method for accomplishing this end and has the additional advantage of providing a precise quantitative analysis of each type of hemoglobin. However, because of the cost of this apparatus, the highly skilled personnel necessary for its operation, and the fact that only one sample can be analyzed in a period of 6 to 20 hours, it is obviously impractical for general use.

Two other laboratory methods for differentiating sickle cell anemia from sickle cell trait erythrocytes have recently appeared. These are the specific agglutination of sickle cell anemia erythrocytes by specific rabbit antiserum (Schneider and Levin, 1950) and the alkali denaturation test (Singer, Chernoff, and Singer, 1951). The latter is not specific for sickle cell anemia, since alkali resistant hemoglobin may be present in other anemias. The former has, in our hands, been found a most convenient and reliable method when a good antiserum was available. Some rabbits offer no difficulties in production of such serum. However, we have recently found (Schneider, unpublished experiments) that many other rabbits produce nonspecific agglutinins difficult to remove by absorption. The production of these nonspecific agglutinins seems to be related to parenteral contact with red cell stroma and bacterial products and to infection. Since substances related to the blood group antigens are widely distributed in nature and since these may offer strong competition to the relatively weak sickle cell antigen, it may be difficult to select rabbits for the production of specific antiserum. If these difficulties can be overcome, this should provide an excellent method to distinguish erythrocytes from sickle cell anemia from those of sickle cell trait. In the meantime, paper electrophoresis of the hemoglobins offers a reliable method that is relatively simple, inexpensive, and rapid, and gives information of a semiquantitative nature about the relative concentrations of normal and sickling hemoglobin in a given patient.

Itano and Neel (1950) have found by means of the Tiselius apparatus, a new inherited abnormality of hemoglobin. This new hemoglobin, tentatively called hemoglobin III (Itano and Neel, 1950) or hemoglobin "C" (Itano, 1951) has been found in combination either with normal or sickling hemoglobin or both. Individuals with a combination of sickling hemoglobin and hemoglobin "C" have a hemolytic anemia of a form milder than sickle cell anemia. Those with a combination of normal hemoglobin and hemoglobin "C" are said to have an asymptomatic carrier state. When erythrocytes of subjects with this state are transfused into normal individuals, they are eliminated more rapidly than are normal erythrocytes (Kaplan, Zuelzer, and Neel, 1951). The incidence of this carrier state is not known, since the only method for detecting it has been by means of the Tiselius electrophoresis apparatus, which is obviously impractical for large-scale surveys. The method of paper electrophoresis offers a practical possibility for this type of survey.

Conclusion

A simple reliable method for paper electrophoresis of human hemoglobin has been described. The method provides an accurate and relatively easy diagnostic test to differentiate patients with sickle cell anemia from those with sickle cell trait, and both of these from the type of sickle cell disease in which sickling hemoglobin is combined with large amounts of hemoglobin "C." This method may also be used to detect the mixture of normal hemoglobin and hemoglobin "C."

Acknowledgments

We are indebted to the following members of The University of Texas Medical Branch: to Miss Jean Stubbins and her staff of the Blood Bank of John Sealy Hospital for their cooperation in obtaining blood samples; to Mrs. Mary Jane Harrell of the Hematology Research Laboratory for the sickling preparations; to Mr. MacDonald Smith and Mr. George LeFeber of the Tissue Culture Laboratory for the illustrations and photography, respectively; and to Mr. John A. Cudd, Mrs. Mary Lou Fraser, and Miss Leedia Marie Greve of the Tissue Metabolism Research Laboratory for the Tiselius Electrophoresis analyses, for technical assistance, and for aid in preparation of the manuscript, respectively.

REFERENCES

Drabkin, D. L.: Spectrophotometric Studies. XIV. The Crystallographic and Optical Properties of the Hemoglobin of Man in Comparison with Those of Other Species. *J. B. C.*, 164: 703–723, 1946.

Durrum, E. L.: A Microelectrophoretic and Microionophoretic Technique. *J. Am. Chem. Soc.*, 72: 2943–2948, 1950.

Flynn, F. V., and P. DeMayo; Microelectrophoresis of Proteins on Filter Paper. *Lancet*, 2: 235–239, 1951.

Itano, H. A.: A Third Abnormal Hemoglobin Associated with Hereditary Hemolytic Anemia. *Proc. Nat. Acad. Sci.*, 37: 775–784, 1951.

Itano, H. A., and J. V. Neel: A New Inherited Abnormality of Human Hemoglobin. *Proc. Nat. Acad. Sci.*, 36: 613–617, 1950.

Kaplan, E., W. W. Zuelzer, and J. V. Neel: A New Inherited Abnormality of Hemoglobin and Its Interaction with Sickle Cell Hemoglobin. *Blood*, 6: 1240–1259, 1951.

Kunkel, H. G., and A. J. Tiselius: Electrophoresis of Proteins on Filter Paper. *J. Gen. Phys.*, 35: 89–118, 1951.

Pauling, L., H. A. Itano, S. J. Singer, and I. C. Wells: Sickle Cell Anemia, a Molecular Disease. *Science*, 110: 543–548, 1949.

Schneider, R. G., and W. C. Levin: Production of Specific Antisera Against Sickle Cell Anemia Erythrocytes. *Proc. Soc. Exp. Bio. and Med.*, 75: 110–114, 1950.

Singer, K., A. I. Chernoff, and L. Singer: Studies on Abnormal Hemoglobins; I. Their Demonstration in Sickle Cell Anemia and Other Hematologic Disorders by Means of Alkali Denaturation. *Blood*, 6: 413–428, 1951.

————: Studies on Abnormal Hemoglobins: II. Their Identification by Means of the Method of Fractional Denaturation. *Blood*, 6: 429–435, 1951.

Wells, I. C., and H. G. Itano: Ratio of Sickle Cell Anemia Hemoglobin to Normal Hemoglobin in Sicklemics. *J. B. C.*, 188: 65–74, 1951.

THE ETIOLOGY OF NUTRITIONAL MUSCULAR DYSTROPHY*

IRA R. TELFORD

The basic intrinsic mechanisms of nutritional muscular dystrophy are obscure. However, several pertinent observations have been reported indicating that the dystrophy, when provoked by tocopherol deficiency, may have circulatory disturbances as some of its etiological factors. Pappenheimer (1931) first described edema associated with cerebellar lesions and collapse of capillaries in vitamin E deficient chicks. In a series of papers Dam and Glavind (1938, 1939, 1939, 1940) reported increased permeability of capillaries and exudative diathesis in vitamin E deficient chicks. Bird and Culton (1940) prevented generalized edema in chicks, held on a synthetic diet, by the addition of alpha-tocopherol to their diet. Edema of the face of young dystrophic rats, suggestive of a "buffalo" head appearance, has been reported by Telford (1942). Fetal death in the uterus of tocopherol-deficient rats is due to a vascular injury with areas of hemorrhage and generalized ischemia (Mason, 1943). Skelton, Shute, Skinner, and Waud (1946) reported that purpura, produced in dogs by administration of stilbestrol, was cured by alpha-tocopherol acetate. Coagulation time in human hemophiliac subjects was decreased when they received injections of vitamin E (Tropeano, 1948). It has been reported that tocopherol prevents hemolysis of rat blood *in vivo* and *in vitro* (Gyorgy and Rose, 1949), and Ames, Baxter, and Griffith (1951) concluded that alpha-tocopherol possesses considerable activity in preventing increased capillary fragility, resulting from irradiation. Pathologic changes in cardiac muscle of E-deficient rabbits described by Gatz and Houchin (1947) were confirmed by Ruppel (1949) who further observed marked changes in the arteries of the extremities.

Pappenheimer (1939) made the pertinent observation later substantiated by us (Telford, 1942), that fibers near the periph-

* From the Department of Anatomy, The University of Texas Dental Branch, Houston. Supported in part by a grant from the U. S. Public Health Service. Received for publication March 4, 1953.

ery of a muscle with the most abundant collateral blood supply enjoy the greatest protection from the dystrophy, and that the muscle lesions might be due to "angiospastic occlusion, causing anoxemia and infarction." The capillaries in dystrophic muscles are empty and collapsed, which is suggestive of angiospasms. If this is true, then such angiospasms may be of neural origin and the adreno-sympathetic system may be involved, causing constriction of blood vessels supplying the muscles, producing anoxemia with subsequent necrosis of the muscle fibers.

Materials and Methods. We investigated the vascular aspect of the problem by varying the amount of blood supplying certain muscles in dystrophic vitamin E deficient rabbits.[1] This was accomplished in each rabbit by one of the following procedures: (1) Unilateral sympathectomy from L_1 through S3 or 4; (2) Resection of a left sciatic nerve as it emerges through greater sciatic foramen; (3) Ligation of left femoral artery as it emerges underneath the inguinal ligament.

The operations were performed with intravenous nembutal anesthesia, under the usual aseptic conditions. The operations were made when animals manifested signs of the dystrophy, namely, marked creatinuria and loss of weight, but showed no significant histological muscle changes as demonstrated by biopsy specimens at the time of the operation. Experience had shown that such animals would begin to show ataxia, partial paralysis and slowed righting reflexes within 2 to 4 days after the onset of the creatinuria. Four to 6 days after the operation, the animals usually became paralyzed and moribund and were, therefore, sacrificed. Comparative histological studies were made of the extensor muscles of both thighs. The unoperated thigh muscles served as the control for the operated side.

Results. In 11 dystrophic rabbits with unilateral sympathectomies, no significant difference in the histological picture of the operated and unoperated thigh muscles was noted. Both thighs showed approximately equal muscle damage. In 14 dystrophic rabbits with their left sciatic nerves removed 4

[1] The vitamin E deficient diet is as follows: casein, 20; cellulose flour, 15; sucrose, 5; corn starch, 41; Brewer's yeast, 10; lard, 3; cod-liver oil, 2; salt mixture (Hubbel), 4. (Daily supplement of 2 mgm. ascorbic acid per animal.)

days before necropsy no histologic differences in either side was observed. Ligation of the left femoral artery of 14 dystrophic rabbits failed to alter to any observable degree the pathological lesions in the quadriceps muscle of the thigh 4 days after the operation.

Discussion. Despite suggestive evidence that nutritional muscular dystrophy, resulting from tocopherol deficiency, may be influenced by the variation of the blood supply to the skeletal muscles, our attempts to increase the blood to the hind limb of the rabbit by removal of the vasoconstrictor fibers, either through unilateral sympathectomies or by sciatic nerve resection, were ineffective in preventing or lessening the muscular lesions. We are reasonably sure that both operations resulted in more blood going to the limb, as evidenced by the increase in skin temperature on the operated side, as measured by skin thermocouples. Our findings, however, do not substantiate the report of Pappenheimer and Geottsch (1940), that resection of the sciatic nerve of suckling rats of vitamin E-low mothers resulted in protection of the gastrocnemius muscle from the usual dystrophic lesions. It would appear from their work that the probable increased blood supply to the denerved limb, resulting from the severance of the sympathetic vasoconstrictor fibers in the sciatic nerve, may have been an important factor in preventing the muscle lesions in young suckling E-deficient rats. We did not duplicate their results in our adult rabbit studies, due perhaps to differences in species response, age and diet discrepancies, and especially the time interval between the operation and death. They reported that protection from the dystrophy was obtained only on those young rats operated upon during the 5th to 17th day. If the nerve resection was delayed until the 18th day or later, there was no protection afforded the operated limb.

Evans (1940) has reported that tocopherol administered to these suckling young of E-low mothers on or after the 15th day did not prevent or delay the typical dystrophic symptoms. If, however, the vitamin was given in adequate dosage any time before the 15th day, complete protection was conferred upon the suckling young rats. Certainly the period between the 15th and 25th day is a critical one as regards need of vitamin E by

young rats. In dystrophic lambs, however, tocopherol therapy is effective in preventing paralysis throughout life. (Willman *et al.*, 1945). The immature transition stage of the suckling rat or lamb cannot be critically compared to the relatively stable condition of the adult rabbits used in our investigation.

The efficacy of our procedures in increasing the blood supply to the muscles, may be questioned by the report of Kemp, Tuttle, and Hines (1947). These workers embedded thermocouples into the denervated gastrocnemius muscle of dogs and found subnormal temperatures soon after the denervation, and these lower temperatures persisted thereafter. Yet, they reported an immediate increase of 189 per cent blood flow in the femoral artery following sciatic nerve resection. This increased blood flow gradually leveled off until an average of 105 per cent flow was noted on the 21st day after the nerve resection, and at the end of 54 days an average flow of 76 per cent of the normal was recorded. Since our rabbits were sacrificed 4 to 6 days after denervation, the operated limb was probably receiving a significant increase in blood during this period. This conclusion was based on the elevation of skin temperature following the operation. We, unfortunately, did not embed thermocouples into the muscles of our animals, so we did not determine the muscle temperature of these animals. How much of the increased blood flow actually reached the muscles might be questioned, yet in reflex vasodilator response studies in man, Wilkins and Eichna (1941) present evidence that this vasodilation does occur in the muscular segments of the limb.

The reduction of the amount of blood going to the left hind limb by ligation of the femoral artery did not bear out the observation of Le Gros, Clark, and Blomfield (1945) or Betz (1947), that the resulting ischemia provoked an additional degenerative response. We could detect no additional increase of muscle nuclei, macrophages, or necrotic muscle masses in the ligated limb. These authors, however, were studying total ligation of all vessels supplying a muscle. We made no attempt to ligate collateral vessels, in fact, we were concerned with reducing, not obliterating the blood supply to the limb. Ligation of the femoral artery in our rabbits did not increase the severity of the lesions. It should be noted, however, that col-

lateral circulation is probably rapidly increased in the thigh after ligation of its main blood supply, and before the full effect of the ligation can express itself by its own degenerative processes, the collateral may have opened up sufficiently to partially cancel the ligation effects.

Conclusions

We conclude that under these experimental conditions of altering the normal blood supply to thigh muscles of the dystrophic rabbit by unilateral sympathectomy, sciatic nerve resections or femoral artery ligation, had no significant effect upon the course of the muscle lesions in dystrophy provoked by alpha-tocopherol deficiency. It seems unlikely that variations in the blood supply to the striated muscles are etiological factors in the production of the muscular lesions observed in nutritional muscular dystrophy in the rabbit.

REFERENCES

Ames, S. R., J. G. Baxter, and J. O. Griffith: Prevention by d-a-tocopherol of increased capillary fragility in rats following irradiation. *Intern. Z. Vitaminforsch.*, 22: 401–7, 1951.

Betz, H.: Sur l'origine musculaire de cellules histiocytaires au cours de la dégénérescence des fibres striées. *Comptes Rendus de la Societe de Biologie*, 141: 1276–1278, 1947.

Bird, H. R., and T. J. Culton: Generalized edema in chicks prevented by d, l-alpha tocopherol. *Proc. Soc. Exp. Biol. and Med.*, 44: 543–547, 1940.

Dam, H., and J. Glavind: Anti-encephalomalacia activity of dl-a-tocopherol. *Nature*, 142: 1157–1158, 1938.

————: Alimentary exudative diathesis, a consequence of E avitaminosis. *Nature*, 143: 810–811, 1939.

————: Alimentary exudative diathesis and its relation to Vitamin E. *Skand. Arch. f. Physiol.*, 82: 299–316, 1939.

————: Vitamin E and capillary permeability. *Naturwissenschaften*, 28: 207, 1940.

Evans, H. M.: The William Henry Welch lectures. I. New light on the biological role of vitamin E. *J. Mt. Sinai Hosp., N.Y.*, 6: 233–244, 1940.

Gatz, A. J., and O. B. Houchin: Histological observation on the vitamin E deficient rabbit heart. *Anat. Rec.*, 97: 337, 1947.

Gyorgy, P., and C. S. Rose: Tocopherol and hemolysis *in vivo* and *in vitro*. *Ann. N. Y. Acad. Sci.*, 52: 231–239, 1949.

Kemp, C. R., W. W. Tuttle, and H. M. Hines: Studies on temperature characteristics, blood flow, and activity in normal and denerved limbs of a dog. *Am. J. Physiol.*, 150: 705–711, 1947.

Le Gros Clark, W. E., and L. B. Blomfield: Efficiency of intramuscular anastomosis, with observation on regeneration of devascularized muscle. *J. Anat.*, 79: 15–32, 1945.

Mason, K. E.: A hemorrhagic state in the vitamin E-deficient fetus of the rat. Essays in Biology in honor of Herbert M. Evans. University of California Press, 401–409, 1943.

Pappenheimer, A. M., and M. Goettsch: Cerebellar disorder in chicks, apparently of nutritional origin. *J. Exp. Med.*, 53: 11–36, 1931.

Pappenheimer, A. M.: The pathology of muscular dystrophy in young rats. *Am. J. Path.*, 15: 179, 1939.

Pappenheimer, A. M., and M. Goettsch: Effect of nerve section upon development of nutritional muscular distrophy in young rats. *Proc. Soc. Exp. Biol. and Med.*, 43: 313–316, 1940.

Ruppel, W.: Changes in the organs of rats in vitamin E deficiency. *Arch. f. exp. Path. u. Pharm.*, 206: 584–601, 1949.

Skelton, R. F., E. V. Shute, H. G. Skinner, and R. A. Waud: Antipurpuric action of a-tocopherol. *Science*, 103: 762, 1946.

Telford, I. R.: Thesis—An histological and functional study of striated muscles in vitamin E deficient rats. George Washington University, Washington, D.C., 1942.

Tropeano, L.: Action of vitamin E on the coagulation of blood. *Progr. Med. Napoli*, 4: 169–170, 1948.

Wilkins, R. S., and L. W. Eichna: Blood flow to forearm and calf; vasomotor reaction: role of sympathetic nervous system. *Bull. Johns Hopkins Hosp.*, 68: 425–429, 1941.

Wellman, J. P., J. K. Loosli, S. A. Asdell, F. B. Morrison, and P. Olafson: Prevention and cure of muscular stiffness (stiff lamb disease) in lambs. *J. Animal Sci.*, 4: 128–132, 1945.

CALLING ATTENTION TO:

Items of Possible Interest To Our Friends

April, 1953

Clinical: HH Anderson, WL Bostick & HG Johnstone sketch *Amebiasis: Pathology, Diagnosis & Chemotherapy* (CC Thomas, Springfield, Ill, 53, 431pp, $11.5). . . . JA Baumann discusses gross aspects of human growth & CA Baud histological (*Schw Med Wchnschr* 83/179,185/Feb 21/53). . . . F Bergman & L Wislicki find toxic doses (0.5 Gm/kg) of nicotinamide cause BP fall, hyperglycemia & tubular oliguria (*Brit J Pharmacol Chemo* 8/48/53). . . . H Droller recommends foliandrin (oleander glucoside) in congestive heart failure with ischemia (*Cardiol* 22/118/53). . . . M Fishbein edits *1953 Medical Progress* (Blakiston, NY, 53, 301pp, $4.5). . . . MA & JW Goldzieher edit *Endocrine Treatment in General Practice* (Springer, NY, 53, 474pp, $8). . . . A Haddow, GM Tinnes & DAG Galton find dimethane sulfonyl oxybutane ("Myerlan") safe & effective in chronic myeloid leukemia orally 4–10 mgm per day (*Lancet* 1/207,208/Jan 31/53). . . . A Haddow describes *Biological Hazards of Atomic Energy* (Oxford Univ Press, NY, 53, 246pp, $8). . . . DA Hamburg & Co emphasize emotional problems of burned patients (*N Eng J Med* 248/355/Feb. 26/53). . . . H Herzog & W Pulver report Wasserman positive virus pneumonia (*Schw Med Wchnschr* 83/227/Mch. 7/53). . . . JH Holines & AV Montgomery discuss thirst as a symptom (*Am J Med Sci* 225/281/53). WF Lever reviews pemphigus (*Med* 31/1/53). . . . A Smith & PL Wermer edit *Modern Treatment: A Guide for General Practice* (Hoeber, NY, 53, 1146pp, $20) . . . . O Spuhler reviews interstitial nephritis (*Schw Med Wchnschr* 83/145/Feb 14/53) . . . Our HG Swann describes principles of resuscitation (*Anesth* 14/126/53) . . . HG Wolff discusses *Stress & Disease* (CC Thomas, Springfield, Ill, 53, 180pp, $5.5) . . . CS Wright & Co describe standardized test for erythrophagocytosis (*J Lab Clin Med* 41/169/53).

General: DW Cory compiles 21 *Variations on a Theme* which is unusual (Greenberg, NY, 53, 448pp, $3.75). R Gelatt offers *Music Makers* (Knopf, NY 53, 312pp, $3.75). WN Kellogg & Co think porpoise sounds are sonar signals (*Science* 117/239/Mch 6/53). C Singer gives prime English translation of *Vesalius on the Human Brain* (Oxford Univ Press, NY, 53, 177pp, $5). MB Shimkin & Co consider experimentation on humans (*ibid*, p 205, Feb 27/53). GR Stewart describes *US 40: Cross Section of USA* (Houghton Mifflin, Boston, 53, 311pp, $5). N(Cybernetics) Wiener looks back at *Ex-Prodigy: My Childhood & Youth* (Simon & Schuster, NY, 53, 310pp, $3.95).

Fundamental: WW Ackerman & RB Johnson conclude energy for viral synthesis comes from oxidative phosphorylation action of host tissue (*J Exp Med* 97/315/53). . . . RJ Barrnett shows sulfhydryl is all tissues except bone, cartilage, mucous, elastic & collagen (*J Nat Canc Inst* 13/905/53). . . . H Becks & HM Evans offer *Skeletal Development of Normal & Hypophysectomized Rat* (Amer Inst Dent Med, Berkeley, Calif, 53, 2 vols,

145 pp, $48. F Bergman & A Shimoni correlate nerve conduction with membrane pH in enzyme buffer system of acetylcholine & esterase (*Biochim Biophys Acta* 10/49/53). R Biggs & Co show platelets, antihemophilic globulin, serum fraction VII, & $CaCl_2$ necessary for thromboplastin formation (*J Physiol* 119/89/53). . . . SS Cohen discusses control of virus synthesis by antimetabolites (*Pediat* 11/89/53). . . . JH Gaddum discusses bioassays & mathematics (*Pharmacol* Rev 5/87/53) . . . AGS Hill & B Cruickshank find 2 antigens in kidney, one in basement membranes gives antibody able to give nephritis (*Brit J Exp Path* 34/27/53). . . . EJ Kempf edits symposium on comparative conditioned neuroses (*Ann NY Acad Sci* 56/141–380/53). . . . N Lahovary offers bioserological methods of human classification (*Science* 117/259/Mch 13/53). . . . A Lazarow designs direct reading microgasometric apparatus (*Lab Invest* 2/22/53). . . . MO Lee edits 1556 abstracts for Federated Societies meeting, Chicago, April 6–10 (*Fed Proc* 12/473pp/53) . . . WK Livingston asks What Is Pain? (*Sci Am* 188/59/53) . . . HG McCann shows fluorapatite formed at all Ca:P ratios below 5ppm fluoride, but CaFl occurs at higher fluoride conc (*J Biol Chem* 201/247/53). . . . AG Ogston & JE Stanier find hyaluronic acid influences lubrication of synovial fluid (*J Physiol* 119/244/53). . . . WO Reinhardt & CH Li find hypophyseal growth hormone can cause arthritis (*Science* 117/295/Mch 20/53) . . . Our EC Rennels & Co find estrogens in males increases adrenals (*Proc Soc Exp Biol Med* 82/304/53) . . . AB Rothballer describes neurosecretion in hypothalamus & neurohypophysis after pain (*Anat Rec* 115/21/53) . . . Our GR Seaman finds thioctic acid required for acyl transfer from pyruvate (*Proc Soc Exp Biol Med* 82/184/53). . . . Our HG Swann & Co find no practical differences in resuscitation methods (*J Appl Physiol* 5/421/53). . . . Note Symposium on *Structural Aspects of Cell Physiology* (Academic Press, NY, 52, 357pp, $7.5) . . . SG Tomlin shows similarity of reticulin & collagen (*Nature* 171/302/Feb 14/53) . . . M Wiedersheim & Co discuss blood & temperature effects of water sol polysacharides (*Arch Exp Path Pharmak* 217/107/53). . . .

MAY, 1953

GENERAL: S Chamberlain delights in *Bouquet de France: An Epicurean Tour of the French Provinces* (Gourmet Distr Corp NY, 53, 619pp, with superb illustrations & recipes, $10). S Freud's complete works announced in 24 vols (Lange Maxwell & Springer, London WC 2, 53,L30) . . . AT Gaul notes *The Wonderful World of Insects* (Photoned by Rinehart, NY, 53, 290pp,$4) . . . B Ghiselin edits *The Creative Progress* with 38 contributors (Univ California Press, Berkeley, 53, 260pp, $6.5) . . . W. Haymaker edits *The Founders of Neurology* with 133 biobibliographical sketches (CC Thomas, Springfield, Ill, 53, 479pp, $10) . . . J Huxley humanizes in *Evolution in Action* (Harper, NY, 53, 182pp, $2.5) . . . T King eulogizes *Water: Miracle of Nature* (Macmillan, NY, 53, 238pp, $3.5) . . . RD Mosier discusses *The American Temper: Patterns of our Intellectual Heritage* (Univ California Press, Berkeley, 53, 306pp, $5) . . . EW Sinnott observes *Two Roads to Truth* (Viking, NY, 53, 280pp $3.5) . . . D Stamp describes *Our Undeveloped World* as answer to starvation

(Faber, London, 53, 266pp, 18s). . . . JR Smythies discusses mescaline phenomena (*Brit J Philos Sci* 3/339/53).

CLINICAL: F Bar approaches therapy of central nervous virus infection (*Arzneimittelfors* 3/103/53) . . . JGG Borst & Co show synergistic action of liquorice & cortisone in adreno-cortical deficiency & AI Card & Co find glycerrhetinic acid (fr liquorice) causes water & salt retention (*Lancet* 1/657,663, Apr 4/53) . . . LJ Borstelmann &WG Klopfer critically review Szondi personality test (*Psych Bull* 50/112/53) . . . KM Bowman & B Engle consider problem of homosexuality (*J Social Hyg* 39/2/53) . . . YH Guinena suggest irritative encephalopathic syndrome as basis for study of epilepsies & psychoses (*J Roy Egypt Med Asso* 36/114/53) . . . J Learmonth discusses ischemia in surgery (*Brit Med J* 1/743/Apr 4/53). . . . G Moros & Co suggest 4 cc 70% diodrast in fluorodensography with photoelectric cell to study cardiovascular conditions (*Am Heart J* 45/495/53) . . . AE Needham offers *Regeneration & Wound Healing* (Methuem, London, 53, 151pp, 8s6d) . . . H Paul surveys *The Control of Communicable Diseases* (J deGraff, NY, 53, 720pp, $9.5) . . . JP Peters reviews edema of acute nephritis (*Am J Med* 14/448/53) . . . FM Rackeman introduces symposium on allergy (*Pract* 170/333–385/53) . . . S Rowlands reviews isotopes as aid to diagnosis (*Edinb Med J* 60/149/53) . . . DA Sadowsky & Co conclude smoking is not etiologically associated with lung cancer (*J Nat Canc Inst* 13/1237/53) . . . JT Scales & Co discuss design of cuirass respirators (*Lancet* 1/671/Apr 4/53) . . . CC Sturgis offers *Hypersplenism: A Clinical Evaluation* (CC Thomas, Springfield, Ill, 53, 97pp, $3) . . . G Waldbott discusses *Contact Dermatitis* (CC Thomas, Sprinfield, Ill, 53, 218pp, $6) . . . S Wieden confirms (*Med J Austral* 1/364/Mch 14/53) HG Kunkel's zinc sulfate turbidity test for liver disease (*Gastroenterol* 11/499/48) . . . HG Wolff relates *Stress & Disease* (CC Thomas, Springfield Ill, 53, 180pp, $5).

PRECLINICAL: A Albert discusses *Selective Toxicity & Chemotherapy* (Methuen, London, 53 132pp, 7s6d) . . . G Ashwell & JH Hickman indicate ATP enzymically active spleen fraction is radiation resistant (*J Biol Chem* 201/651/53) . . . OJ Brock emphasizes base binding power of serum proteins in osmoregulation of body fluids (*Scand J Clin Lab Invest* 6/9/53) . . . A Butenandt discusses biochemistry of genes (*Naturwis* 40/91/53) . . . E Geiringer & DC Hardwick show increase in skin histamine after remote injury (*J Physiol* 119/410/53). JR Grassi describes *The Grassi Substitution Test for Measuring Organic Brain Pathology* (CC Thomas, Springfield, Ill, 53, 75pp, $2.5) . . . CH Gray discusses *The Bile Pigments* (Methuen, London, 53, 331pp, 9s6d) . . . MA Heimrich Jr show antihistaminics increase barbital narcosis (*Arch Internat Pharmaco* 92/444/53) . . . DJ Ingle & BL Baker note *Physiological & Therapeutic Effects of Corticotropin (ACTH) & Cortisone* (CC Thomas, Springfield, Ill, 53, 172pp, $4). D Keilin & EF Hartree indicate cytochrome oxidase is part of catalytic system in Pasteur reaction (*Nature* 171/413/Mch 7/53) . . . W v Robertson & R Schwartz show ascorbic acid needed for collagen formation (*J Biol Chem* 201/689/53) . . . J Sacks describes *Isotopic Tracers in Biochemistry & Physiology* (McGraw-Hill, NY, 53, 383pp, $8.5). RL Saunders notes cadaver preservation by plastics (*Anat Rec* 115/43/53) . . . HM Vander

Eecken & RD Adams indicate protective function of many brain meningeal arterial anastomoses (*J Neuropath Exp Neurol* 12/132/53) . . . W Wirth analyses cholinergic action of insecticide diethyl thio phosphate of ethyl thioglycol, "systox" (*Arch Exp Path Pharmakol* 217/144/54) . . . SA Waksman edits *Neomycin* with chapters by our CS Livingood & E Poth (Rutgers Univ Press, New Brunswick, NJ, 53, 219pp, $5).